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INHIBITION OF HYPOTHALAMIC, MEDULLARY AND REFLEX VASOMOTOR RESPONSES BY CHLORPROMAZINE

BY

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From the Department of Pharmacology, School of Tropical Medicine, Calcutta

(RECEIVED APRIL 27, 1954)

In the course of an extensive pharmacological investigation Courvoisier, Fournel, Ducrot, Kolsky and Koetschet (1953) noticed that chlorpromazine (3-chloro - 10(3′ - dimethylaminopropyl)phenothiazine; 4560 R.P.; "Largactil"; "Megaphen") abolished the pressor reflex elicited by electrical stimulation of the central end of the vagus nerve. In the dose used for the demonstration of this effect, chlorpromazine also antagonized the pressor effects of adrenaline and noradrenaline. Dasgupta, Mukherjee and Werner (1954) observed that chlorpromazine very effectively suppressed the sham rage of diencephalic animals in doses considerably lower than those used by Courvoisier *et al.*; it was therefore concluded that chlorpromazine could block certain hypothalamic responses and that severance of cortico-hypothalamic tracts made such an action apparent. Other observations on central effects of chlorpromazine—such as the potentiation of the activity of narcotics (Courvoisier *et al.*, 1953; Zipf and Alstaedter, 1954) and of analgesics, block of conditioned reflexes (Courvoisier *et al.*, 1953) and behaviour changes with simultaneous alterations of the electroencephalogram (Das, Dasgupta and Werner, 1954)—made us wonder whether a central depressant action participated in the inhibition of vasomotor reflexes. Experiments were therefore undertaken to find whether, and under what conditions, chlorpromazine could block vasomotor responses centrally.

METHODS

Chlorpromazine was injected intracisternally into rhesus monkeys, anaesthetized with allobarbitone (50 mg./kg.). The blood pressure was recorded from the femoral artery, and carotid sinus reflexes were elicited by clamping both carotid arteries.

In cats under chloralose anaesthesia (60–80 mg./kg., i.v.) or decorticated under ether, pressor responses were elicited by electrical stimulation of the sciatic nerve with an induction coil, and by stimulation of medullary and hypothalamic pressor areas with a Horsley-Clark stereotaxic instrument. In the latter, the electrodes, insulated except for the tip, conveyed rectangular pulses of 0.5 msec. duration, at 100/sec.

In all experiments on cats the brain specimens were fixed in formol saline and the electrode position was verified histologically (Weil-stain). In most experiments under chloralose, and in all experiments on decorticate cats, artificial respiration was used throughout after paralysis of the spontaneous respiration with decamethonium (50–100 μg./kg. i.v., repeated when necessary).

RESULTS

Intracisternal Application of Chlorpromazine.—In a dose of 0.25–0.5 mg. per animal chlorpromazine caused a fall of blood pressure on intracisternal injection. Depending on the initial pressure, a reduction of the mean arterial pressure by 30 to 50 mm. Hg was observed in 4 such experiments on rhesus monkeys (5–7 kg. body weight).

The pressor reflex elicited by clamping both common carotid arteries was always abolished for at least 40 min. and then gradually returned to its original height in the course of 2–3 hr. The pressor response to intravenously injected adrenaline was not altered during the hypotension and reflex inhibition.

Experiments in Cats under Chloralose Anaesthesia.—In 14 such experiments, attempts were made to determine the effect of chlorpromazine on the sciatic pressor reflex, and on the pressor response caused by direct electrical stimulation of medullary and hypothalamic pressor areas. It was not always possible to obtain good responses to all three stimuli in the same animal, but at least two of the three tests could usually be carried out with satisfactory responses as regards height of blood-pressure rise (30–50 mm. Hg) and constancy of the response on repeated stimulation before administration of the drug. The posterior hypothalamus was aimed at with the Horsley-Clark instrument, and the actual site of stimulation was later verified histologically. The medullary pressor points selected for our experiments were, for reasons of

easier accessibility, chosen around the lateral wings of the caudal reticular formation, dorso-lateral to the inferior olivary nucleus (Alexander, 1946).

The results with chlorpromazine were not very uniform. In 8 experiments, 0.5 mg. kg. i.v. abolished reflex as well as direct medullary and hypothalamic pressor responses. In other experiments, however, doses even higher than 1 mg./kg. did not significantly lower the pressor responses. To assure better uniformity of the experimental conditions and to avoid complications arising from respiratory inhibition due to the central or reflex stimulation, the animals were, in some experiments, paralysed with decamethonium and artificially ventilated; however, the results with chlorpromazine did not gain in uniformity. These experiments, therefore, provide no evidence for a specific central vasomotor reflex blocking action, because the reduction of the pressor effects could be accounted for by the peripheral adrenolytic action of chlorpromazine.

In a few experiments the electrode tips were found to be situated in the pedunculi cerebri, or in the rostral parts of the pons, instead of the hypothalamus; no correlation between sensitivity of the pressor response to chlorpromazine and the site of the stimulated area was observed.

In view of our previous observation (Dasgupta *et al.*, 1954) that the central depressant activity of chlorpromazine greatly increases after decortication, the following experiments were undertaken.

Experiments on Decorticate Cats.—Hypothalamic and medullary pressor areas were stimulated in decorticate cats. In addition, the sciatic pressor reflex was used as a test response in some experiments. In all eleven experiments of this group, a more specific effect was now obtained with chlorpromazine. In doses of 50–100 μg./kg. intravenously, the pressor responses of reflex and central origin were completely abolished usually for several hours (Fig. 1); indeed, complete recovery was seldom obtained. The suppression of the pressor response was always accompanied by a large fall of blood pressure (20–40 mm. Hg), which also persisted for several hours. This marked hypotensive action was in sharp contrast to the lack of any significant effect of chlorpromazine on the blood pressure in animals with an intact central nervous system, even if administered in doses more than 10 times higher. Cannon and Britton (1925) have given evidence for a hypersecretion from the adrenal medulla in decorticated animals, which could be abolished by an additional transection at the level of the mid-brain; obviously the central blocking effect of chlorpromazine will appear more effective against the background of such a high sympathetic activity. In this connexion, reference can also be made to several of our experiments in which, following decortication, regular blood pressure waves were observed for about 15–30 min.; these animals were paralysed with decamethonium and artificially ventilated. Chlorpromazine in doses of 50–100 μg./kg. abolished the rhythmic blood pressure waves completely and at the same time lowered the mean arterial pressure by 30–50 mm. Hg (Fig. 2).

Discussion

The observations reported here show that, in certain circumstances, a central depressant effect of chlorpromazine on vasomotor tone and reflex activity can be demonstrated. The conditions necessary for the demonstration of this effect are either intracisternal application of the drug in anaesthetized, or intravenous administration in decorticated, animals. Particularly in the latter, a very pronounced suppression of vasomotor reflexes and of pressor effects of central origin can be detected in doses which are so low as to be devoid of any peripheral actions (Courvoisier *et al.*, 1953). The

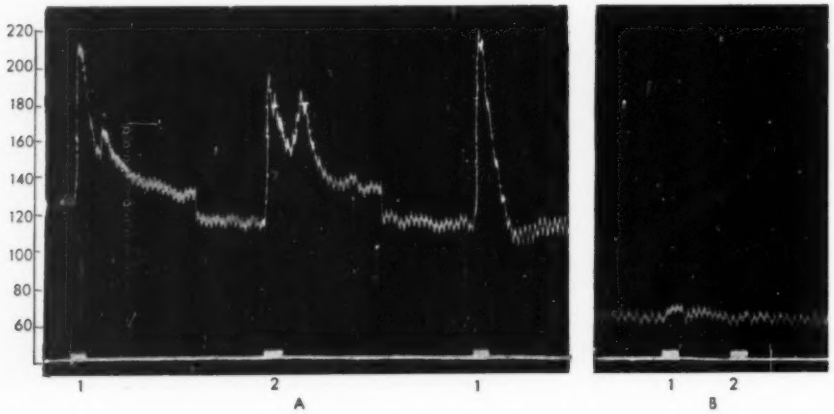

Fig. 1.—Decorticated cat (3.4 kg.); blood pressure in mm. Hg. Signals indicate periods of stimulation (7 sec.) of (1) hypothalamus (4.5 V) and (2) medulla (1.5 V). A before, and B 4 min. after, intravenous injection of 100 μg./kg. chlorpromazine.

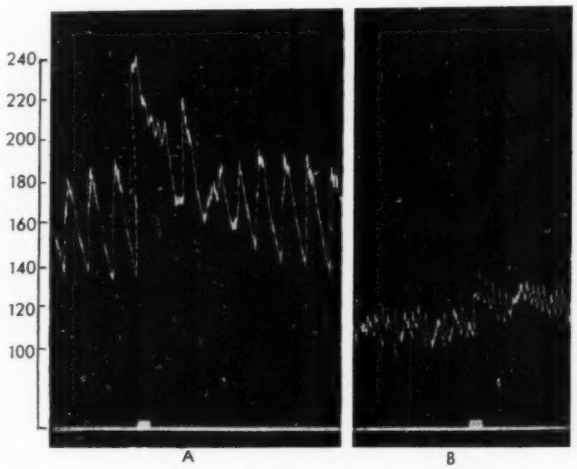

FIG. 2.—Decorticated cat (3.0 kg.); blood pressure in mm. Hg. Signals indicate periods of stimulation of the sciatic nerve (5 sec.; induction coil). A before, and B 6 min. after, intravenous injection of 100 μg./kg. chlorpromazine.

results in decorticate animals supplement our previously reported findings regarding the effect of chlorpromazine on the behaviour of decorticate cats: it is apparent that the effects of direct electrical stimulation of the hypothalamus are suppressed by chlorpromazine in the same way as the signs of sham rage when evoked by reflex stimulation (Dasgupta *et al.*, 1954).

Comparable observations with morphine reveal a different pattern of activity: only the skeletal motor components of sham rage are reduced or abolished in therapeutic doses (Wikler, 1944), whereas effects of direct hypothalamic stimulation are not suppressed (Masserman, 1939). However, before assigning to chlorpromazine any central activity peculiar to this drug only, it will be necessary to compare the pattern of activity of other drugs with central depressant actions under similar experimental conditions.

SUMMARY

1. Intracisternal injection of chlorpromazine to rhesus monkeys causes a fall of blood pressure and suppression of carotid sinus reflexes.

2. Pressor responses elicited by electrical stimulation of hypothalamic or medullary pressor areas, and the sciatic pressor reflex, are specifically abolished in decorticated cats by chlorpromazine in doses of 50–100 μg./kg.

3. Cats under chloralose anaesthesia are considerably less susceptible to such blocking actions of chlorpromazine.

The authors desire to express their appreciation to Dr. John Harper for stimulating suggestions, and to May & Baker, Ltd., for the chlorpromazine.

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Brit. J. Pharmacol. (1954), **9**, 392.

COMPARISON OF SOME PHARMACOLOGICAL PROPERTIES OF CHLORPROMAZINE, PROMETHAZINE, AND PETHIDINE

BY

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(RECEIVED JULY 15, 1954)

The pharmacological actions of chlorpromazine have been described in detail by Courvoisier, Fournel, Ducrot, Kolsky, and Koetschet (1953). This substance was introduced into anaesthetic practice by Laborit and Huguenard (1951) and is given together with promethazine and pethidine, so that it is impossible to know what contribution each makes to the joint effect. We have therefore compared their pharmacological properties quantitatively as well as qualitatively.

The properties tested were the power to reduce body temperature; the effect on skeletal muscle stimulated directly and through the nerve; the chronic toxicity; the power to potentiate (*a*) pentobarbitone, (*b*) morphine; the anti-adrenaline action; the anti-acetylcholine action; the antihistamine action; and the local anaesthetic action.

METHODS

Body Temperature

Eight male mice were used in each experiment, their rectal temperatures being recorded by inserting a lubricated thermocouple into the rectum. This thermocouple was connected to a moving coil galvanometer and the cold junction thermocouple was placed in a constantly stirred water bath at 25° C. The mice were held in position by fastening the tail with adhesive tape to one end of a wire and turning the other end into a loop round the chest. Each mouse was placed in a small celluloid cage of hemispherical cross-section. After half an hour to an hour their temperatures remained steady, and then four mice were used as controls and were injected with saline, while the other four were injected with the substance being tested. The mean difference in temperature between the two groups of mice before the injections was taken as zero and allowance was always made for this difference in calculating the mean temperature-difference between the test mice and the controls recorded at 15-min. intervals.

Skeletal Muscle

The sciatic-gastrocnemius preparation of the cat was used as described by Dale and Gasser (1926). The muscle was stimulated indirectly through the sciatic nerve by rectangular voltage pulses and directly through the muscle by means of induction shocks from a Lewis rotary contact breaker. The rates of stimulation were adjusted so that they were both about 8 or 9/min., and the muscle was continuously stimulated by each method alternately at 1 min. intervals. Injections were made into the right iliac artery close to the bifurcation of the abdominal aorta.

The phrenic nerve-diaphragm preparation of the rat was also used. The experimental procedure was the same as that described by Bülbring (1946), the nerve being stimulated by a pair of platinum electrodes submerged in the organ bath (50 ml.).

Anti-adrenaline Action

The anti-adrenaline actions of chlorpromazine, promethazine and pethidine were compared on three preparations—the vessels of the rabbit ear, the blood pressure of the spinal cat, and the smooth muscle of the rabbit uterus.

(a) *Vessels of Rabbit Ear.*—The central artery of the rabbit ear was cannulated and injections were made into it by means of the device described by Gaddum and Kwiatkowski (1938). A Gaddum drop-timer was used to record the changes in vascular tone, and single injections of the substance being tested were made. A dose of each substance was found which reduced the effect of adrenaline to one-tenth of the initial effect, so that after an injection of the anti-adrenaline substance a dose of adrenaline 10 times greater than the initial dose was required to produce the same constriction.

(b) *Blood Pressure of Spinal Cat.*—A dose of adrenaline (about 10 μg.) was found which produced a reasonably large rise in blood pressure. The anti-adrenaline actions of chlorpromazine, promethazine and pethidine were then compared by finding the dose of each drug which reduced the effect of adrenaline to about one-third of its initial value. There were difficulties in an accurate estimation of chlorpromazine, since the vessels were rapidly desensitized to its action and since this desensitization lasted for several hours.

(c) *Rabbit Uterus.*—The anti-adrenaline actions of the three drugs were compared by finding the dose of each substance which reduced the effect of adrenaline to one-half of the initial effect.

Anti-acetylcholine Action

The anti-ACh actions of chlorpromazine, promethazine and pethidine were compared on two preparations, the guinea-pig ileum and the pupil of the mouse eye. The action of chlorpromazine on salivary secretion was also studied.

(a) *On Ileum.*—A piece of guinea-pig ileum was suspended in oxygenated Locke at 36° C. and a dose of ACh was found which produced reasonably large submaximal contractions. The anti-ACh actions of chlorpromazine, promethazine and pethidine were compared on this preparation by exposing the gut to a certain dose of each drug for 60 sec., and finding the dose such that twice the original dose of ACh then produced a contraction of equal height to the standard.

(b) *On Mouse Pupil.*—Chlorpromazine, promethazine and pethidine were tested on the mouse pupil for mydriatic activity by the method of Pulewka (1932). The mice (18–28 g.) were injected intraperitoneally with 0.2 ml. of the solution being tested and the effect on the size of the pupil was determined under a binocular microscope fitted with a scale in one eyepiece. The diameter was measured before and 20 min. after injections of chlorpromazine, promethazine and pethidine, by which time the optimal mydriatic effect had been reached.

(c) *On Salivary Secretion.*—The anti-ACh action of chlorpromazine was compared with that of atropine on salivary secretion. The method used was that described by Bülbring and Dawes (1945). A steady flow of saliva was usually produced by an intravenous infusion of a solution containing adrenaline (5–10 μg./ml. depending on the blood pressure of the cat) and carbachol (5–10 μg./ml.) at a rate of about 0.5 ml./min.

Antihistamine Action

The antihistamine actions of chlorpromazine, promethazine and pethidine were compared on two preparations, the guinea-pig ileum and the guinea-pig bronchioles.

(a) *On Ileum.*—Their antihistamine actions on the ileum were compared in exactly the same way as the anti-ACh actions. The dose of each substance was found which reduced the effect of histamine to one-half of the initial effect.

(b) *On Bronchioles.*—The bronchial tone was recorded by the method described by Konzett and Rössler (1940). A guinea-pig was anaesthetized with a 25% solution of urethane and the lungs were artificially respired by a Starling pump, which drove air into the trachea. The excess air which did not enter the trachea operated a float-recorder which recorded a vertical line on a kymograph. When the bronchioles constricted the lever rose, so that the length of the vertical line was a measure of the constriction. A dose of histamine, which caused a reasonably large bronchoconstriction, was injected intravenously at regular intervals until a uniform response was obtained. The antihistamine actions of chlorpromazine, promethazine and pethidine were then compared by finding the doses which caused about a 50% reduction in the response to the standard dose of histamine.

Local Anaesthetic Action

The local anaesthetic activities of chlorpromazine, promethazine and pethidine in 0.3%, 0.1% and 0.05% (w/v) concentrations were compared by the intracutaneous weal method described by Bülbring and Wajda (1945). Four injections were made into shaven areas on the back of a group of guinea-pigs, two at the front and two at the back, and, since the sensitivity of the different areas on the back of a pig varies considerably, all three concentrations of the three drugs were tested on each area. The reaction to six pin-pricks was determined at each site every 5 min. and the number of failures to respond to 36 pricks was a measure of the degree of anaesthesia.

Results

Body Temperature

The effects of subcutaneous injections of chlorpromazine (1 mg./kg.), promethazine and pethidine (30 mg./kg.), and hexamethonium (5 mg./kg.) on body temperature were compared and the results are shown in Fig. 1. Observations were also made

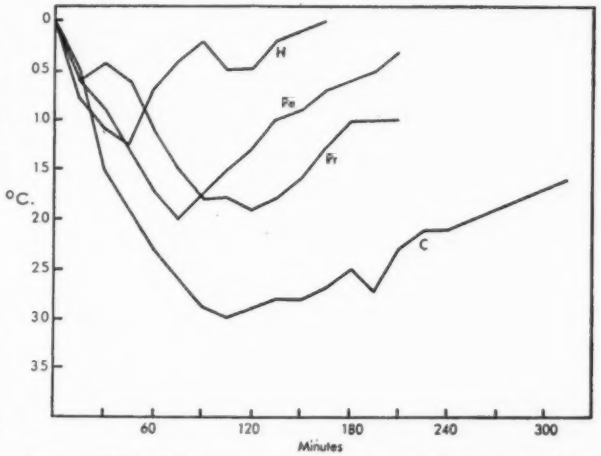

FIG. 1.—Effect of drugs on the body temperature of mice. Abscissae: time in min.; ordinates: mean temperature difference between test mice and controls. The results for each drug are the means of experiments with 12 control and 12 test mice, except hexamethonium, where 4 control and 4 test mice were used. H = hexamethonium, 5 mg./kg.; Pe = pethidine, 30 mg./kg.; Pr = promethazine, 30 mg./kg.; C = chlorpromazine, 1 mg./kg.

with a higher dose of chlorpromazine (3 mg./kg.) when the mean temperature-difference between the injected mice and controls was as much as 5.7° C. after 3 hr. and still 2° C. after 7 hr. from the time of injection. Promethazine in a dose of 3 mg./kg. had virtually no effect on body temperature.

Action on Striated Muscle

In the sciatic-gastrocnemius preparation of the cat, chlorpromazine caused a slight initial increase

in the size of contractions, both to direct and indirect stimulation, followed by a gradual decrease. The size of the contractions to direct stimulation always fell away more slowly than those to indirect stimulation. These effects are shown in Fig. 2, where it is seen that a dose of 4.6 mg./kg. eliminated completely the response to indirect stimulation after 75 min.

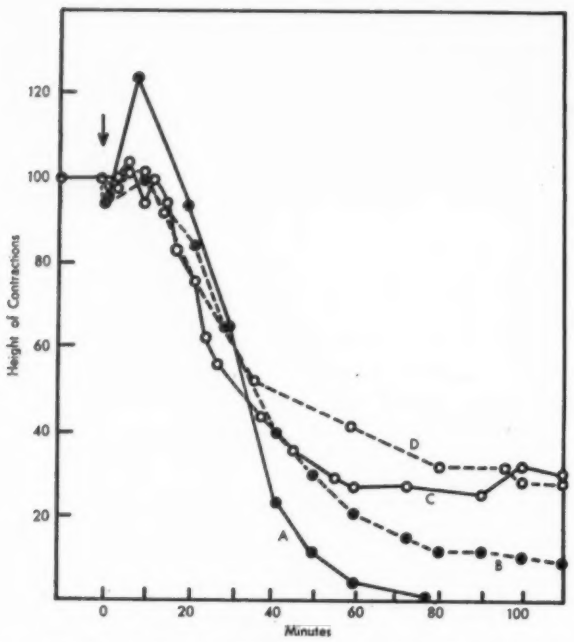

FIG. 2.—Effect of chlorpromazine (injected into the aortic bifurcation) on the twitch tension of the cat gastrocnemius to direct and indirect stimulation. Abscissae: time in min.; ordinates: height of contractions expressed as a percentage of the initial height. Injections of chlorpromazine made at arrow. The full lines indicate the response to indirect stimulation and the broken lines to direct stimulation. A and B are the responses after 4.6 mg./kg., and C and D after 3.1 mg./kg.

Similar effects were observed with promethazine, which had about 50% of the action of chlorpromazine, and pethidine, which had only about 10%. These figures are approximations, since there were wide variations in the 9 different animals used. The effects were not due to failure of the circulation, for in several experiments where the blood pressure was raised from a rather low level to about the original height by an infusion of pituitary extract (10 u./50 ml.) no alteration in the gradual decline of the contractions was observed. Furthermore, the effects were not due to fatigue, since in one experiment the gastrocnemius muscle was stimulated for 3 hr. before any injection was made and throughout this time always gave about the same response to the electrical stimuli (Fig. 3). Between (*b*) and (*c*) an injection of pethidine (20 mg./kg.) was made, and thereafter the picture obtained was exactly the

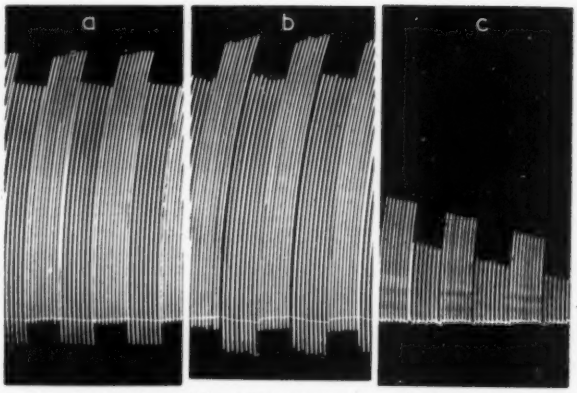

FIG. 3.—Contractions of the cat gastrocnemius. Single supramaximal shocks, 8 or 9 per min.; 0.7 millisecond duration. (*a*) Direct and indirect stimulation. (*b*) The same after 3 hr. continuous stimulation. (Note that there is no sign of fatigue.) (*c*) 40 min. after injection of pethidine (20 mg./kg.) into the iliac artery.

same as in other experiments. In one or two experiments it became necessary to apply artificial respiration.

The effect of nalorphine on this paralysing action of pethidine was investigated, but in only one experiment out of three was a moderate degree of recovery observed. In others the nalorphine had no effect. All attempts to counteract the action of chlorpromazine with nalorphine were unsuccessful.

In the phrenic nerve-diaphragm preparation similar results were obtained. In experiments with indirect stimulation alone, chlorpromazine in doses of about 100 μg. caused a slight increase in the muscle contractions. In doses of about 200 μg., there was a very rapid decrease and the contractions were abolished within 20 min. Promethazine behaved in a similar way except that a rather higher dose (400 μg.) was required to abolish the contractions (Fig. 4). Very much larger doses of pethidine were necessary to produce these effects; 2 mg. produced an increase in the contractions and about 10 mg. produced a decrease. These figures for pethidine agree closely with those of Dutta (1949). The contractions usually recovered after washing, showing that the effects of the chlorpromazine, promethazine, and pethidine were reversible. This was of particular interest, since the contractions in the cat sciatic-gastrocnemius preparation were never found to recover within 6 hr. of observation. Tests were made in 8 preparations.

Chronic Toxicity

The acute toxicity of chlorpromazine in mice has been studied by several workers, but very few details are available concerning chronic toxicity. The effect of chlorpromazine, promethazine, and pethidine on the growth of young rats was therefore studied. In each experiment, rats (35–55 g.) were

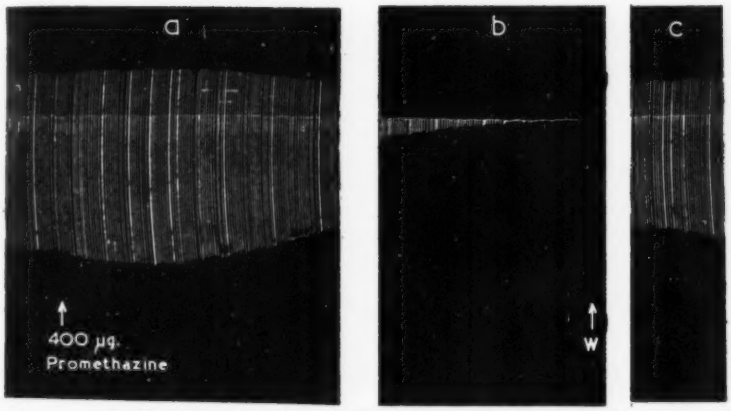

FIG. 4.—Rat phrenic nerve-diaphragm preparation in Tyrode solution, 37° C., 50 ml. bath. Single supramaximal shocks (7 per min., 0.7 msec. duration) through the phrenic nerve. (*a*) 400 μg. promethazine at arrow. (*b*) Response to stimuli abolished after 50 min. Change of bath fluid at W. (*c*) 25 min. later. The size of contractions had returned almost to their original height.

injected subcutaneously twice daily and their rate of growth, in comparison with control rats which received saline injections, was followed for at least a week. For the first 2 or 3 days the rats receiving chlorpromazine, promethazine, or pethidine in sufficiently low doses were unaffected and gained in weight by an amount approximately equal to that of the control group. In rather higher doses their mean weight remained constant or even fell. After 3 days the mean weight usually increased at the same rate as the controls (Fig. 5). There were occasional deaths after the third day, however, when the general condition of the rats was apparently improving and the typical drowsiness observed after the first few injections of chlorpromazine and promethazine was getting less and less. Pethidine produced an increase in excitability rather than drowsiness.

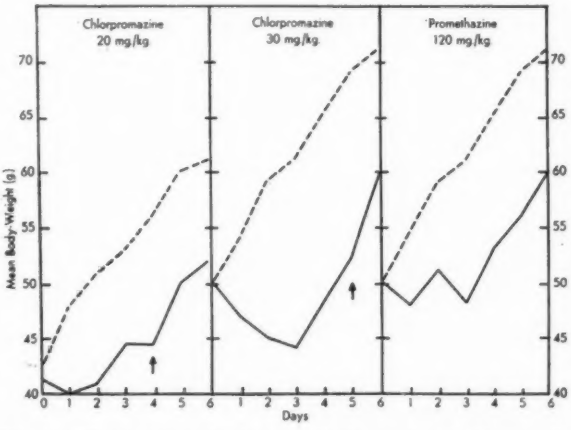

FIG. 5.—Effects on growth of rats. Abscissae: time in days; ordinates: mean body weight in g. Broken lines indicate the growth rate of controls and full lines the growth rate of test rats. At the arrows one test rat was found dead in its cage. Note that the inhibition of growth lasted for only 2 or 3 days.

From Table I it is seen that a dose of 30 mg./kg. of chlorpromazine for the first 4 days caused a retardation in growth similar to that produced by a dose of 150 mg./kg. of promethazine or pethidine. Thereafter all 3 substances had no further retarding action. It therefore appeared that chlorpromazine was about 5 times more toxic than promethazine or pethidine. Although 40 mg./kg. of chlorpromazine killed 3 out of 4 rats when given as a single or as a divided dose, this higher dose was tolerated by a group of 8 rats which had previously received a daily dose of 20 mg./kg. for one week. This fact, together with the decrease in drowsiness in the course of the injections, suggested that the rats acquired tolerance towards these drugs.

TABLE I

EFFECT ON GROWTH OF RATS (35–55 G. INITIAL WEIGHT)

Drug	Total Daily Dose (mg./kg.)	Mean Difference in Weight from Controls (g.)		No. of Deaths
		0–4 Days	0–6 Days	
Chlorpromazine ..	10	−2	−2	0/8
	20	−11	−8	1/15
	30	−19	−14	1/4
	40			6/8
	40*			
	40†	−6	−10	0/8
Promethazine ..	40*	0	0	0/4
	120	−12	−11	0/4
	150	−11	−12	3/8
Pethidine	100*	+6		0/4
	150	−8	−11	4/8
	200	−11		3/4

* One injection daily.
† These received a daily dose of 20 mg./kg. for one week previously.

Potentiation of Morphine and Pentobarbitone

The effect of chlorpromazine, promethazine, and pethidine on the action of morphine and of pentobarbitone was compared.

Morphine.—The analgesic action of morphine (1.5 mg./kg.), chlorpromazine, promethazine, and pethidine (all 10 mg./kg.), and then of morphine together with each of the above three drugs in the same doses, was tested by the application of electrical stimulation to the mouse tail (Grewal, 1952). All injections were given subcutaneously. The results are shown in Table II, from which it is seen that by this method the analgesic action of morphine was not potentiated by either chlorpromazine, promethazine, or pethidine. Nor indeed did the

TABLE II
ACTION ON MORPHINE ANALGESIA

Drug	No. of Mice	% Showing Analgesia
Morphine (1·5 mg./kg.)	20	10
Chlorpromazine (10 mg./kg.)	40	70
,, +morphine	40	40 (80)
Promethazine (10 mg./kg.)	40	25
,, +morphine	40	37·5 (35)
Pethidine (10 mg./kg.)	40	40
,, +morphine	40	35 (50)

The figures in brackets are those to be expected for an additive effect.

effect, except with promethazine, seem to be additive. The results quoted in Table II are for observations 15 min. after the injection of the substances. By this time the optimum effect had apparently been reached, for even after 30 or 60 min. the percentage of mice showing analgesia was not significantly different.

Pentobarbitone.—A group of 40 mice was injected intraperitoneally with pentobarbitone (50 mg./kg.), and the mean duration of sleep in minutes was observed. The mice were placed on a warm table and were considered to be asleep as long as they remained lying on their sides. Observations were also made on further groups of mice which were given chlorpromazine (10 mg./kg. and 3 mg./kg.), promethazine and pethidine (both 10 mg./kg.) in addition to the pentobarbitone. The drugs were all given subcutaneously, except pentobarbitone, which was given intraperitoneally. This was done because a precipitate was formed when the solutions were mixed. The results are shown in Table III.

TABLE III
ACTION ON PENTOBARBITONE HYPNOSIS

Drug	No. of Mice	Mean Duration of Sleep (min.)
Pentobarbitone (50 mg./kg.)	40	28·3
Chlorpromazine (3 mg./kg.)+pentobarbitone	80	39·5
,, (10 ,,)+ ,,	40	154
Promethazine (10 mg./kg.)+pentobarbitone	40	40·2
Pethidine (10 mg./kg.)+pentobarbitone ..	40	39·5

Chlorpromazine in a dose of 10 mg./kg. had a potentiating action on pentobarbitone and produced a nearly fivefold increase in the duration of sleep.

Anti-adrenaline Action

The approximate equipotent ratios on the three preparations used are shown in Table IV. Chlor-

TABLE IV
ANTI-ADRENALINE ACTION

Drug	Vessels of Rabbit Ear	Blood Pressure of Spinal Cat	Smooth Muscle of Rabbit Uterus
Chlorpromazine	1	1	1
Promethazine..	4	33	10
Pethidine ..	32	41	10,000

promazine in doses of the order of 1–10 mg. injected in the spinal cat reduced the response to adrenaline to about 25% of the initial value, this being the mean value in 7 cats. The effect of noradrenaline, however, was only slightly diminished, as shown in Fig. 6.

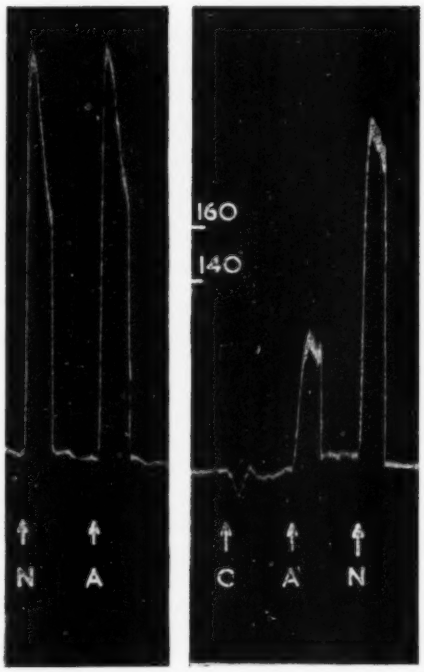

FIG. 6.—Effect of chlorpromazine on the adrenaline and noradrenaline blood-pressure rise in the spinal cat. N=8 μg. noradrenaline, A=15 μg. adrenaline, and C=10 mg. chlorpromazine. It can be seen that chlorpromazine in a dose which greatly reduces the effect of adrenaline has scarcely any effect on an equipotent dose of noradrenaline.

The effect of chlorpromazine on the blood-pressure rise after splanchnic stimulation was investigated before and after adrenalectomy. Before adrenalectomy there was the usual initial rapid rise due to constriction of vessels in the viscera, followed by a secondary less rapid rise due to liberation of adrenaline from the adrenal glands. After 5 mg. of chlorpromazine the blood-pressure rise was reduced by nearly 50% and the secondary rise was abolished, because of the anti-adrenaline action of the chlorpromazine. After about an hour, however, the blood-pressure rise had returned to its original

level. After adrenalectomy two doses of 5 mg. of chlorpromazine (given within 10 min.) then had virtually no effect; indeed, a slightly larger rise in blood pressure was observed.

In further experiments chlorpromazine was found to reduce the rise of blood pressure following nicotine in the spinal cat, when the adrenal glands were present.

Anti-acetylcholine Action

The approximate equipotent ratios are shown in Table V—for the guinea-pig ileum in terms of chlorpromazine=1, and for the mouse pupil in terms of atropine=1. On the mouse pupil chlorpromazine in doses as high as 20 mg./kg. (400 μg./mouse) showed no mydriatic activity whatsoever.

TABLE V
ANTI-ACETYLCHOLINE ACTION

Drug	Guinea-pig Ileum	Mouse Pupil
Chlorpromazine	1	No action
Promethazine	0·13	60
Pethidine	2·7	120
Atropine	Not tested	1

In experiments on the salivary flow, chlorpromazine produced a fall of blood pressure and showed an atropine-like action in inhibiting the salivary flow. This inhibition was not due to the blood-pressure fall, since after a minute or two the salivary flow returned to its original level, while the blood pressure did not recover. In 4 observations on 2 cats chlorpromazine appeared to be about one-thirtieth as active as atropine. We did not test promethazine and pethidine for ability to inhibit salivary secretion, but Schaumann (1940) reports that pethidine is more than 200 times weaker than atropine.

Antihistamine Action

The approximate equipotent ratios of the three drugs on the bronchioles and on the ileum are shown in Table VI. Promethazine is more potent on both preparations, particularly the bronchioles, than either chlorpromazine or pethidine, and its action also lasts very much longer.

TABLE VI
ANTIHISTAMINE ACTION

Drug	Guinea-pig Ileum	Guinea-pig Bronchioles
Chlorpromazine	1	1
Promethazine	0·33	0·01
Pethidine	40	150

Comparison of Antihistamine and Anti-acetylcholine Actions

The relative antihistamine and anti-acetylcholine actions of each substance on the guinea-pig ileum were compared by finding the dose required to reduce the response to histamine and acetylcholine by nearly 100%. The results are shown in Table VII. The antihistamine action of chlorpromazine is seen to be much stronger than its anti-acetylcholine action (Fig. 7).

TABLE VII
WEAKNESS OF ANTI-ACETYLCHOLINE ACTION, RELATIVE TO ANTIHISTAMINE ACTION TAKEN AS=1

Chlorpromazine	1 : 33
Promethazine	1 : 4
Pethidine	1 : 1·5

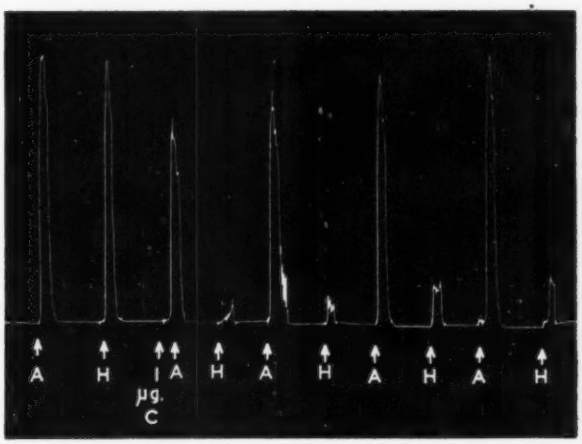

FIG. 7.—The antihistamine and anti-acetylcholine action of chlorpromazine on the guinea-pig ileum. A=0.1 μg. acetylcholine, H=0.4 μg. histamine, and C=chlorpromazine. Note that the antihistamine action of chlorpromazine is much greater and longer-lasting than the anti-acetylcholine action.

Local Anaesthetic Action

The activity of chlorpromazine was found to be 1.2 times greater than that of promethazine and 2.1 times greater than that of pethidine. Their relative local anaesthetic activities are shown in Fig. 8. Chlorpromazine, promethazine, and pethidine were tested for ability to induce plexus anaesthesia in frogs by the method of Bülbring and Wajda (1945). All three were active in concentrations of about 0.1–0.2%, chlorpromazine again being more active than promethazine or pethidine.

DISCUSSION

Chlorpromazine, promethazine, and pethidine were all found to lower the body temperature of mice, chlorpromazine being more than 30 times as effective as promethazine or pethidine. A similar

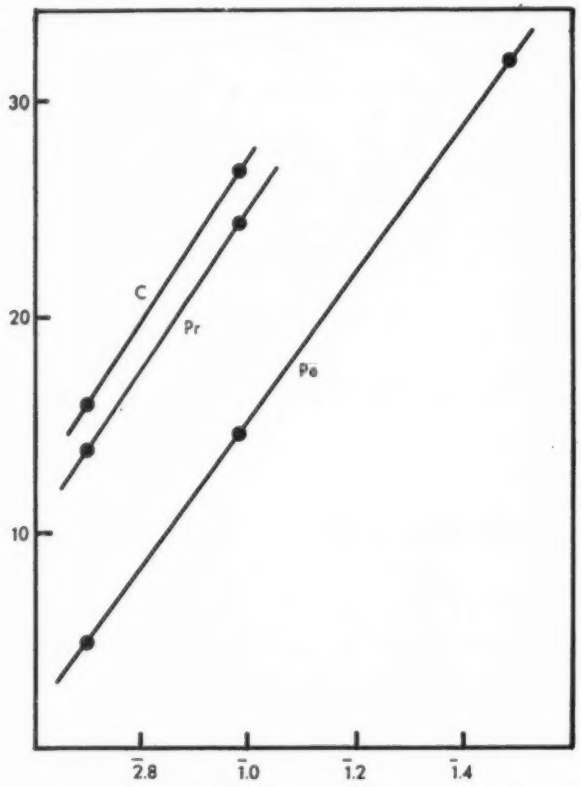

FIG. 8.—Local anaesthetic activity. Abscissae: log concn.; ordinates: degree of anaesthesia expressed as the mean failure to respond to 36 pricks in 30 min. The points represent the mean of observations on 15 guinea-pigs. C=chlorpromazine, Pr=promethazine, Pe=pethidine.

result was obtained by Courvoisier *et al.* (1953) comparing chlorpromazine and promethazine.

When the gastrocnemius muscle of the cat was stimulated, either directly or indirectly through the sciatic nerve, all three drugs depressed the response of the muscle. The doses required to produce these effects varied, but chlorpromazine was always the most effective. From the fact that both direct and indirect stimulation failed to have any appreciable effect after the muscle was paralysed, it was evident that the action was not curare-like but was a direct action on the muscle.

Since body temperature is maintained to a large extent by the normal tonus of skeletal muscles, any interference with the power to contract will affect heat production as well as the tonus. Our findings clearly show that chlorpromazine, promethazine, and pethidine have a depressant effect on skeletal muscles, and we therefore suppose that this action is at least partly responsible for the lowering of body temperature. There is also the possibility that these substances influence the chief centres for the integration of temperature control in the hypothalamus, although, as yet, there is no experimental evidence to support this view. It may appear surprising that none of the authors describing the action of chlorpromazine, promethazine, and pethidine in human subjects mentioned an effect on the skeletal muscles. It must be remembered, however, that in clinical practice the usual reduction in body temperature by these drugs, without external cooling, is not more than 1–2° C., so that the effect on skeletal muscles is presumably not great enough to be detected by routine clinical methods. Although the doses we used in our experiments on the cat were not very much higher than those used clinically, they were injected intra-arterially close to the muscle, and it is also likely that there may be considerable variation in species sensitivity. Paralysing effects on skeletal muscles, sufficient to be noticed by the clinician, may therefore be obtained only with much higher doses.

It has been stated by some anaesthetists that chlorpromazine owes its action on body temperature to a ganglion-blocking effect. We tested hexamethonium for power to reduce body temperature in a dose of 5 mg./kg., and found that it produced only a slight fall, much less than that caused by 1 mg./kg. chlorpromazine.

Our experiments showed that all the three substances investigated when given in sufficiently high doses inhibited the growth of young rats during the first three days. After this time the rate of growth was not affected, but during the period of our observations the mean weight of the test groups never reached that of the controls. These results can be interpreted in two ways: either the rats lost weight through loss of appetite as a consequence of drowsiness, or they lost weight because of an inhibitory effect on the metabolic rate. The first possibility is ruled out for pethidine, since pethidine produced excitability in the rats rather than drowsiness. If the second possibility is correct this might throw some light on the much-discussed problem as to whether chlorpromazine has a depressant action on metabolism or not.

Some authors, Courvoisier *et al.* (1953), Ducuing and Rieunau-Serra (1953), Irmer and Koss (1953), Peruzzo and Forni (1953), Staehelin and Kielholz (1953), Zettler (1953) and Laborit (1954), state that a reduction in the metabolic rate occurs in tissue slices as well as in whole organisms. Another group, Decourt (1953), Grosse-Brockhoff (1954), Peters and Lehr (1954), and Willems (1954), in experiments on plants, animals, normal and hyperthyroid human subjects, find that within the range of therapeutic doses no reduction in metabolism can be observed. Filk, Ritter, Stürmer, and

Loeser (1954) found in their experiments on rats a decrease in O_2 consumption during the first fourteen hours, but after continuous administration of chlorpromazine for several days the O_2 consumption of the animals returned to its normal level and remained unchanged. Our results seem to confirm these observations. Staehelin and Kielholz (1953) and Dundee, Gray, Mesham, and Scott (1953) also found a small reduction in the basal metabolic rate in human subjects. That Grosse-Brockhoff (1953) and Willems (1953) did not find any depression was probably because they used very much smaller doses.

Decourt (1953) and Peters and Lehr (1954) observed irreversible damage to plant cells, bacteria, moulds and infusoria only in very high concentrations. Weaker solutions never seemed to affect the cells directly. A " histoplegic " action, i.e. a depressant effect on every individual cell (Decourt, 1953, and Laborit, 1953), is therefore not very likely so far as therapeutic doses are concerned. From observations that the metabolism of brain sections is depressed by chlorpromazine (Peruzzo and Forni, 1953) it cannot be concluded that the metabolism of the whole body is also reduced.

Furthermore, the assumption that an effect on metabolism is the consequence of the fall in body temperature does not seem to be correct, since Juvenelle, Lind, and Wegelius (1952) have found that a pronounced inhibition of metabolic processes is only achieved when the temperature is reduced to below 25° C. So great a fall of temperature is not known to have been produced by these drugs alone when patients have survived.

In previous papers it has been reported that chlorpromazine potentiates the analgesic action of morphine both in animals and in human subjects (Courvoisier *et al.*, 1953; Wirth, 1954; Zettler, 1953; and Reckless, 1954), and we have studied the relative power of chlorpromazine, promethazine and pethidine in potentiating this effect. We followed the method of Grewal (1952) and were unable to detect an action of this kind, although the number of animals used in these experiments was certainly great enough to show any potentiating effect. Not even an additive effect was observed, however, except with promethazine. The property of potentiating the action of anaesthetics has been described in many publications (Courvoisier *et al.*, 1953; Dietmann, 1954; Dundee *et al.*, 1953; Staehelin and Kielholz, 1953; Zettler, 1953; and Zipf and Alstaedter, 1954), and all authors agree that the amount of anaesthetic required to produce surgical anaesthesia is considerably reduced. We investigated this potentiating action on pentobarbitone, and in the doses used promethazine and pethidine showed a slight potentiation but very much less than that of chlorpromazine.

On all three preparations (vessels of the rabbit ear, blood pressure of the spinal cat, and smooth muscle of the uterus) an anti-adrenaline action of chlorpromazine, promethazine, and pethidine was found, chlorpromazine always being the most potent, followed by promethazine and pethidine. There is a considerable variation between the equipotent ratios found by the three methods, but this is hardly surprising in view of the widely differing preparations.

Chlorpromazine itself causes a fall of blood pressure. This has been described by all workers who have used it experimentally or clinically. Our experiments on the cat's blood pressure with noradrenaline injections and stimulation of the splanchnic nerve before and after adrenalectomy provide further evidence that chlorpromazine possesses an anti-adrenaline action but very little anti-noradrenaline action. We never succeeded in producing a clear reversal of the adrenaline pressor effect, yet with sufficiently high doses of chlorpromazine it was possible to abolish almost completely the adrenaline rise of blood pressure. At this stage noradrenaline still showed a pressor effect in doses previously equipotent to adrenaline. It should therefore be possible by injecting noradrenaline to raise the blood pressure in patients when the fall is the result of chlorpromazine action.

Courvoisier *et al.* (1953) and others have described the anti-acetylcholine action of chlorpromazine. We studied the action of the three substances individually, and found that on the gut promethazine was about 8 times more active than chlorpromazine, which was about twice as active as pethidine. On the mouse pupil our results were similar to those of Friebel, Flick, and Reichle (1954), promethazine again being more potent than pethidine, whereas chlorpromazine showed no mydriatic activity at all. Nieschulz, Popendiker, and Sack (1954), who worked with a substance structurally related to chlorpromazine (P.391), made similar observations; there was an anti-acetylcholine action on the blood pressure, salivation, and peristalsis of the gut, but only a very weak one on the mouse pupil.

The effect of chlorpromazine, promethazine, and pethidine on bronchial and salivary secretion is recognized as an advantage by all surgeons (Zettler, 1953; Irmer and Koss, 1953; Koss, 1954; and Dundee *et al.*, 1953), since it reduces the number of postoperative pulmonary complications. In psychiatry, however, this side-effect is rather troublesome, producing dryness of the mouth and a burning sensation (Segerath, 1954). We tried to verify this

action and compared it quantitatively with atropine by the method of Bülbring and Dawes. Chlorpromazine only was tested, and, although about 30 times weaker than atropine, a definite inhibitory effect on the salivary flow of the cat, produced by a carbachol infusion, was always observed.

Promethazine has been known since 1946 as a powerful antihistaminic (Halpern and Ducrot, 1946; Viaud, 1947), and was used in conjunction with chlorpromazine and pethidine mainly on account of this property, since some authors believe that neither chlorpromazine nor pethidine possesses any antihistaminic activity (Irmer and Koss, 1953; Smith and Fairer, 1953). We therefore made a quantitative comparison of the antihistamine activity of the three substances, all of which were found to be antihistaminics, the most potent being promethazine, then chlorpromazine and finally pethidine, which is very weak indeed. This order is the same in the experiments on the gut as on the guinea-pig bronchioles, although there is a considerable difference in the actual ratios obtained by the two methods. On the gut, promethazine is 3 times stronger than chlorpromazine, which is 40 times stronger than pethidine, whereas on the bronchioles promethazine is 100 times stronger than chlorpromazine, which is 150 times stronger than pethidine.

The comparison of the antihistamine and anti-ACh actions of chlorpromazine, promethazine, and pethidine on the guinea-pig ileum reveals that all three are stronger antihistaminics than anti-ACh substances—chlorpromazine by more than 30 times, promethazine by 4 times, and pethidine by 1.5 times.

The local anaesthetic actions of chlorpromazine, promethazine, and pethidine were compared. By the intracutaneous weal method in guinea-pigs and measurement of the plexus anaesthesia in frogs, chlorpromazine was the most active and promethazine more active than pethidine. Our results therefore agree with those of Courvoisier *et al.* (1953).

In conclusion, it appears that chlorpromazine possesses some actions in greater degree than promethazine or pethidine, but that all three substances may be regarded as sharing the same type of pharmacological action (Dutta, 1948, 1949).

Summary

1. The following pharmacological properties of chlorpromazine, promethazine, and pethidine were compared:

(1) Action in causing a fall of body temperature.
(2) Action in causing paralysis of striated muscle.
(3) Chronic toxicity.
(4) Potentiation of morphine and pentobarbitone.
(5) Anti-adrenaline action.
(6) Anti-acetylcholine action.
(7) Antihistamine action.
(8) Local anaesthetic action.

2. Chlorpromazine always proved to be more active than promethazine or pethidine, except in its anti-acetylcholine and antihistamine actions.

3. Promethazine was the most active anti-acetylcholine and antihistamine compound; pethidine, on all the preparations tested, was consistently the weakest.

4. Chlorpromazine alone was tested for its anti-noradrenaline action and its effect on salivary flow. It was found to have only a very slight anti-noradrenaline action and it exerted an atropine-like action on salivary flow.

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THE ACTION OF CHLORPROMAZINE ON DIENCEPHALIC SYMPATHETIC ACTIVITY AND ON THE RELEASE OF ADRENOCORTICOTROPHIC HORMONE

BY

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(RECEIVED AUGUST 28, 1954)

Chlorpromazine exerts a peculiar type of sedative action in man and in animals, potentiates and prolongs the action of analgesics and hypnotics, antagonizes excitation by alcohol and nikethamide, and protects animals from shock (for reference see Courvoisier, Fournel, Ducrot, Kolsky and Koetschet, 1953). To explain some of these effects, it has been suggested that the drug has an inhibitory action on sympathetic diencephalic centres (Laborit and Huguenard, 1951 ; Delay and Deniker, 1953) and that it prevents the release of adrenocorticotrophic hormone (ACTH) in conditions of stress (Aron, Chambon, and Voisin, 1953), an action which might also be interpreted as an inhibition of hypothalamic activity. The object of this paper is to investigate these possibilities.

METHODS

Hypothalamic Activity in Cats.—Six cats were subjected to left adrenal denervation, under ether anaesthesia, with aseptic precautions ; on the left side, both splanchnic nerves were severed and the first three lumbar sympathetic ganglia extirpated. In two of the cats, the left superior cervical ganglion was removed a fortnight later. Between two and three weeks after the first operation, the drugs under investigation were injected subcutaneously ; between 4 and 5 hr. after the injection of the first drug, the cats were rapidly anaesthetized with chloroform, bled to death, and their hypothalamus and adrenal glands removed for examination.

Morphine HCl and nalorphine HBr were injected as 2% solutions in 0.9% NaCl ; chlorpromazine was given as 0.15% solution, since higher concentrations have an irritant action.

Estimation of hypothalamic noradrenaline and of the amines of the adrenal medulla was carried out by biological assay on extracts subjected to paper chromatography ; the separated amines were eluted from the paper and the eluates assayed on the rat's blood pressure (urethane anaesthesia ; pretreatment with 2 mg./kg. atropine sulphate and 16 mg./kg. hexamethonium bromide intravenously). In some experiments the results of the adrenaline estimations were checked on the rat's uterus. All details of the methods have been reported earlier (Vogt, 1954).

Blood Pressure and Pupil Size of Dogs.—The action of chlorpromazine as an antagonist of adrenaline was followed on a dog anaesthetized with chloralose. The femoral blood pressure was recorded and observations were made of the effects on pupil size produced by the injection of drugs and by faradic stimulation of the preganglionic fibres of the severed cervical sympathetic trunk.

Experiments on the Adrenal Cortex of Rats.—The release of ACTH in conditions of stress was followed in rats by adrenal ascorbic acid estimations according to the method of Roe and Kuether (1943). One set of experiments was carried out in anaesthesia (1.75 g. urethane/kg. subcutaneously), and " operative stress " used to stimulate the release of ACTH. When the rat was anaesthetized an abdominal incision was made, the intestine exposed, and the mesentery handled for a period of 2 min. The wound was sewn up, the rat replaced in a warm box and killed, while still deeply anaesthetized, by severing the neck 1 hr. later ; the adrenals were then removed for ascorbic acid estimations. The effects obtained in normal rats were compared with those seen in rats injected subcutaneously with chlorpromazine 30 min. or 3 hr. before the anaesthetic.

In a second group of experiments, the fall in adrenal ascorbic acid following a subcutaneous injection of adrenaline was compared under normal conditions and 3 hr. after the subcutaneous injection of chlorpromazine. All rats used in this experiment had been accustomed to handling by being given subcutaneous injections of 0.9% NaCl for a preliminary period of one week.

RESULTS

Hypothalamic Activity in Cats.—For testing central sympathetic activity use was made of the fact that morphine, given to cats, causes stimulation of the hypothalamic sympathetic centres. This can readily be measured by denervating one adrenal and estimating the difference in amine con-

tent between the innervated and denervated (resting) gland elicited by an injection of morphine. A second index of sympathetic hypothalamic stimulation is the fall in the noradrenaline content of the tissue observed when the stimulus has been acting over a period of several hours (Vogt, 1954). If chlorpromazine was able to antagonize a central sympathetic stimulation, the effects of morphine in depleting the stores of amines in the innervated adrenal and of noradrenaline in the hypothalamus might be inhibited.

Table I illustrates the results. The first cat was used as a control and injected with chlorpromazine only. As might be expected, there was no secretion of adrenaline from the innervated adrenal, and the hypothalamic noradrenaline was not significantly different from normal. Cats 2, 3, and 4 were given 40 mg./kg. of morphine 45 min. after a first dose of 15 mg./kg. chlorpromazine; a second dose of chlorpromazine (10 mg./kg.) was injected about 2 hr. after the morphine. Hypothalamic noradrenaline was low, and there was secretion of amines from the innervated medulla; the figures are not different from those of the last row, which represent previous observations on cats given the same dose of morphine but no chlorpromazine.

Thus chlorpromazine, given in a high dose, had no antagonistic effect on the diencephalic stimulation of the sympathetic centres produced by morphine. In another experiment, this result was compared with the effect of a proved morphine antagonist, nalorphine, on the hypothalamic stimulation produced by the same dose of morphine. Cats 5 and 6 (Table I) were given an injection of morphine 12 min. after the subcutaneous administration of nalorphine. The fall in hypothalamic noradrenaline was completely prevented in both cats; a small stimulation of the innervated adrenal medulla was seen in cat 5, which had the smaller dose of nalorphine, whereas this effect was abolished in cat 6, which was given the larger dose. Suppression of the clinical signs too was more complete in cat 6, which showed no abnormality until nearly 5 hr. after the nalorphine: when it was picked up in order to be anaesthetized with chloroform, there was sudden excitation and tremor, indicating that the effect of the nalorphine was beginning to subside. Cat 5, in contrast, showed salivation and some muscular twitching throughout the experiment, though the animal was much quieter than would have been expected as a result of morphine alone.

The clinical picture after chlorpromazine followed by morphine was varied: cat 2 showed no muscular twitches and no motor excitement; instead, it was sitting still or lying down with its head drooping to one side. In cats 3 and 4 muscular twitching and tremor were not suppressed by chlorpromazine as they had been in cat 2. One short fit of convulsions was seen in cat 4.

One of the cats vomited, a fact which is of interest in view of the excellent anti-emetic action of chlorpromazine in the dog given apomorphine (Courvoisier *et al.*, 1953).

A striking sign, in view of the adrenolytic action of chlorpromazine, was, however, a dilatation of the pupils, whereas the nictitating membranes were relaxed. This was first seen in cat 2 and was obviously caused by the morphine (which is a mydriatic in cats), since the chlorpromazine control (cat 1) had narrow pupils and relaxed nictitating membranes.

In order to determine whether the mydriasis was a sign of circulating adrenaline, or was due to some other cause, the next two experiments (Nos. 3 and 4) were carried out on cats in which the left superior cervical ganglion had been re-

TABLE I

HYPOTHALAMIC NORADRENALINE, AND SECRETION FROM INNERVATED ADRENAL MEDULLA, AFTER SUBCUTANEOUS MORPHINE ALONE AND IN COMBINATION WITH CHLORPROMAZINE OR NALORPHINE

Cat No.	Chlorpromazine* (mg./kg.)	Nalorphine HBr (mg./kg.)	Morphine HCl (mg./kg.)	Hypothalamic Noradrenaline			Amines‡ in Innervated Adrenal Medulla (% of Denervated Medulla)	
				μg./g. Fresh Tissue	Mean (μg./g.)	% of Normal†	Individual Expts.	Mean
1	25	0	0	1·3		94	100	
2	25	0	40	0·8			75	
3	25	0	40	1·2	0·98	68	44	59
4	25	0	40	0·8			59	
5	0	25	30	1·4		101	78	
6	0	37·5	40	1·4		101	100	
Mean of 5	0	0	40		0·92	67	40, 57§	

* Given in two doses, interval 2½ hr. † The normal figure (a mean of 29 cats) is 1.38 μg./g. fresh tissue (Vogt, 1954). ‡ Adrenaline and noradrenaline in the adrenal medulla were estimated separately and added together. § 2 cats only.

The cats had been subjected to denervation of the left adrenal 2–3 weeks previously. Cat 1 was killed 5 hr. after the first injection of chlorpromazine; the other cats were killed 4½ hr. after the injection of morphine.

moved one week previously. The nictitating membranes of both cats remained relaxed throughout the experiment, but dilatation of both pupils—and, more strikingly, of the previously narrower, denervated pupil—occurred, particularly towards the end of the experiment. The obvious interpretation was that the pupils responded to circulating medullary amines and that the adrenolytic effect of chlorpromazine was not very powerful.

Antiadrenaline Effect.—The unexpected mydriasis in response to circulating adrenaline (and noradrenaline) in cats given chlorpromazine prompted a reassessment of the adrenolytic effect of chlorpromazine. Since the substance is credited with a ganglion-blocking action, its effect on transmission in a sympathetic ganglion was also tested. In the cats, response to circulatory medullary amines had been noticed as early as 80 and 95 min. after chlorpromazine in a dose of 15 mg./kg. An acute experiment was therefore carried out on a dog anaesthetized with chloralose, and the responses of blood presssure and pupil to injected adrenaline and noradrenaline, and to faradic stimulation of the preganglionic sympathetic fibres in the neck, were observed.

Between 5 and 15 min. after a first intravenous dose of chlorpromazine (1.5 mg./kg.), the effect of adrenaline (4 μg./kg.) on the blood pressure was reversed and that of the same dose of noradrenaline reduced; the mydriatic effects on the pupil had ceased. Preganglionic stimulation of the sympathetic chain, however, was as effective on the pupil as before. Half an hour after the administration of chlorpromazine, the effect of adrenaline began to recover, and a second, higher dose of chlorpromazine (3.0 mg./kg.) was injected. Adrenaline reversal on the blood pressure again ensued, but the threshold to electrical stimulation of the sympathetic chain remained unaltered. Two hr. 20 min. later, pressor and mydriatic effects of adrenaline were back to normal. The effect of sympathetic stimulation, hitherto unchanged, was then abolished by an injection of hexamethonium bromide (2.7 mg./kg.).

It follows from this experiment that the adrenolytic effect of chlorpromazine need not be as prolonged as is often supposed. As with other adrenaline antagonists, the effects of noradrenaline are more resistant to the drug than those of equal doses of adrenaline, and the effects of sympathetic stimulation are more resistant still. In addition, with the dosage (total of 4.5 mg./kg.) used, there was no sign of inhibition of transmission at the sympathetic ganglionic synapse. The fairly short duration of the adrenaline antagonism explains the observation on cats premedicated with chlorpromazine, in which pupillary dilatation in the sympathetically denervated eye occurred after a dose of morphine causing secretion of the adrenal medulla.

Release of ACTH in Stress.—The first part of this work dealt with possible effects of chlorpromazine on centrally initiated secretion by the adrenal medulla. The second part deals with the question whether the drug influences the stimulation of the adrenal cortex elicited by noxious or other stimuli capable of releasing ACTH.

In a first series of experiments, "operative stress" was employed in view of the fact that chlorpromazine protects animals from traumatic shock. The "stress" consisted in anaesthetizing the rats by subcutaneous injection of urethane, carrying out a laparotomy and handling the intestine. The results are shown in Table II.

In Groups 1–3 chlorpromazine was given in a dose of 10 mg./kg. and the operation was carried out 3 hr. later. The rats were killed and the adrenals removed at 4½ hr. The mean ascorbic acid content of the adrenals of Group 1, given chlorpromazine but not operated on, was 321 mg./100 g., and that of the rats operated on without pretreatment (Group 2) was 212 mg./100 g.

TABLE II

THE EFFECT OF CHLORPROMAZINE ON THE ASCORBIC ACID CONTENT (MG./100 G. FRESH ADRENAL) OF THE ADRENALS OF RATS UNDERGOING AN ABDOMINAL OPERATION

Group	No. of Rats	Treatment			Killed at	Adrenal Ascorbic Acid. Mean ± S.E. of Mean
		At Zero Hr.	At 30 min.	At 3 hr.		
1	7	Chlorpromazine, 10 mg./kg.	—	—	4½ hr.	321 ± 9·6
2	6	—	—	Urethane followed by operation	4½ ,,	212 ± 9·5
3	7	Chlorpromazine, 10 mg./kg.	—	Urethane followed by operation	4½ ,,	218 ± 12·4
4	6	0·9% NaCl	—	—	2 ,,	316 ± 12·4
5	6	Chlorpromazine, 15 mg./kg.	—	—	2 ,,	225 ± 10·0
6	6	Chlorpromazine, 15 mg./kg.	Urethane followed by operation	—	2 ,,	236 ± 6·9

Chlorpromazine was injected subcutaneously as 0·1 or 0·15% solution in saline.
The differences between the ascorbic acid values of Groups 2, 3, 5, and 6 are not significant.

When operation and chlorpromazine were combined (Group 3), the value was 218 mg./100 g.; thus no protection had been afforded the adrenals by preceding the operative stress with chlorpromazine.

In order to obviate the objection that the dose was too low or the timing inadequate, we examined the effect of increasing the dose to 15 mg./kg. and shortening the interval between injection and operation to 30 min. The mean ascorbic acid concentration of Group 5 (chlorpromazine alone) was 225 mg./100 g. Practically the same figure was obtained in Group 6, in which chlorpromazine was combined with the operation. Nearly the same value had been found in Group 2, subjected to the operation only. Saline controls (Group 4) gave a mean ascorbic acid content of 316 mg./100 g. Obviously, the higher dose of chlorpromazine itself acted as a stress of about the same severity as the operation. There is no simple additive effect of multiple stressing stimuli on the depression of adrenal ascorbic acid. Thus the fact that the combination of drug injection and operation, each of which are about equally potent in releasing ACTH, does not produce a greater fall in ascorbic acid than each procedure alone cannot be taken as proof—though it may suggest—that the injection has rendered the organism less susceptible to the effect of the operation.

Though inhibition by chlorpromazine of ACTH release by operative stress could not be demonstrated, the possibility remained that, perhaps as a result of the adrenolytic properties of the drug, release of ACTH in response to administration of adrenaline might be diminished. This possibility was examined in the next series of experiments. A special precaution was, however, necessary. It will be seen from Table II that the rats which had been given a subcutaneous injection of saline (Group 4) showed an adrenal ascorbic acid content of 316 mg./100 g., which is nearly 25% below normal. This response to the injection of saline, which is an effect of emotion, was undesirable in experiments in which conscious animals were to be subjected to several subcutaneous injections. It can be abolished by accustoming the rats to injections; this was done, in all rats used in the next experiment, by injecting 0.9% NaCl twice daily for a week before carrying out the final test with drugs. Table III shows the results. The duration of all experiments was 4½ hr. The "trained" rats, killed 4½ hr. after an injection of saline, had an adrenal ascorbic acid content of 409 mg./100 g.; when the saline was replaced by 10 mg./kg. chlorpromazine, there was a fall to 323 mg. When, 2½ hr. after the chlorpromazine, adrenaline (200 μg./kg.) was injected, there was a further significant fall in ascorbic acid; if the initial injection of chlorpromazine was omitted, the response to the same dose of adrenaline was a little but not significantly larger. It follows that a dose of chlorpromazine, large enough to cause some release of ACTH by itself, is nevertheless unable to inhibit appreciably the pituitary response to a dose of adrenaline administered 2½ hr. later, at a time when, to judge from the fall in rectal temperature, some, at least, of the actions of chlorpromazine are still present.

TABLE III

ASCORBIC ACID (MG./100 G. FRESH ADRENAL) IN THE ADRENALS OF "TRAINED" RATS INJECTED SUBCUTANEOUSLY WITH DRUGS AND KILLED AT 4½ HR.

Group	No. of Rats	Treatment	Adrenal Ascorbic Acid. Mean ± S.E. of the Mean
1	11	0·9% NaCl at zero hr. ..	409 ± 19
2	23	Chlorpromazine at zero hr. ..	323 ± 16
3	23	Chlorpromazine at zero hr. Adrenaline 200 μg./kg. at 2½ hr.	286 ± 7·8
4	23	Adrenaline 200 μg./kg. at 2½ hr.	270 ± 7·2

Chlorpromazine (10 mg./kg.) was injected as 0.1% solution in 0.9% NaCl; the same volume of 0.9% NaCl without chlorpromazine was injected into the control rats of Group 1.

The differences between Groups 1 and 2, 2 and 3, 2 and 4 are significant ($P < 0.01$), but not that between 3 and 4 ($P < 0.1$, > 0.05).

DISCUSSION

The clinical picture of morphine poisoning in the cat is modified by premedication with chlorpromazine; actions of chlorpromazine, such as muscular weakness and relaxation of the nictitating membranes, occur side by side with a somewhat damped manifestation of the effects of morphine —salivation, vomiting, tremors, excitement, and, very occasionally, convulsions. The characteristic central sympathetic stimulation by morphine is not inhibited by chlorpromazine. In contrast, premedication with a true morphine antagonist, nalorphine, abolishes all clinical signs of morphine poisoning, and prevents the central sympathetic stimulation which results in a fall in hypothalamic noradrenaline and in a depletion of amines from the stores of the innervated adrenal gland.

The observation that a peripheral sign of circulating medullary amines, mydriasis, occurred in the denervated pupil of cats treated with chlorpromazine and morphine led to the revision of the view that the peripheral adrenolytic action of chlorpromazine need last for many hours. Confirmation was obtained by observations on the dog, in which no vestige of adrenolytic action was

found 140 min. after 4.5 mg./kg. chlorpromazine given intravenously, and partial recovery had occurred much earlier.

In the French clinical literature, chlorpromazine is sometimes classed among the ganglion-blocking agents (Laborit and Huguenard, 1951). In their experimental study, Courvoisier *et al.* (1953) demonstrated that large doses have a mild inhibitory action on transmission in the vagal ganglia of the heart. No corresponding effect in the sympathetic system was found by Reuse (1954), who compared the effects on the nictitating membrane of stimulating the preganglionic and the postganglionic fibres of the superior cervical ganglion of the cat. In complete agreement with our results on the dilatation of the dog's pupil, Reuse found that even large doses of chlorpromazine did not impair transmission in the ganglion. Reuse also showed that sympatholytic in contrast to adrenolytic action required very high doses (above 5 mg./kg.) of chlorpromazine, and the same observation was made by us in the dog.

The experiments on rats, intended to investigate whether adrenocortical response to stress was inhibited by chlorpromazine, failed to demonstrate such an action; it was, however, necessary to choose a dosage and timing that would minimize interference by the release of ACTH caused by chlorpromazine itself. If no more than 10 mg./kg. were administered and 4½ hr. allowed to elapse between injection of drug and ascorbic acid estimations, some depletion of adrenal ascorbic acid was caused by the chlorpromazine, but not enough to obscure the greater depletion due to the operative stress. This dose, given 3 hr. before the operation, did not inhibit the adrenal response to the second stress. It may be argued that larger doses would have produced such an inhibition; but, as they cause a depletion of adrenal ascorbic acid equal to that of the operation, the determination of the share to be attributed to each stressing agent when both are superimposed is impossible, and interpretation of the results would necessarily be ambiguous. Recently, Georges and Cahn (1953) have also come to the conclusion that administration to rats of a "lytic cocktail" containing approximately 11 mg./kg. chlorpromazine did not prevent the release of ACTH in operative stress, provided the body temperature was above 27° C. Contrary claims by Aron, Chambon and Voison (1953), who used doses of chlorpromazine of 10–50 mg./kg. in order to prevent the adrenal ascorbic acid fall caused by unilateral adrenalectomy, are possibly due to the use of very small numbers of rats and the lack of unoperated controls treated with the drug alone. Georges and Cahn (1953) describe a fall in eosinophils 4 hr. after an injection of 22 mg./kg. chlorpromazine, and Filk and Loeser (1954) report a loss of adrenal lipids a few hours after feeding chlorpromazine. Both phenomena indicate a release of ACTH in response to this drug.

It seemed possible that, even if chlorpromazine did not prevent operative stress from releasing ACTH, it might prevent adrenaline from doing so, at least as a result of its peripheral adrenolytic action. Here again, dosage and timing had to be chosen so that there was only a moderate depletion of ascorbic acid by the chlorpromazine alone. Under these conditions, the results were not impressive. The ascorbic acid depletion by adrenaline alone was just very slightly greater than that caused by adrenaline after premedication with chlorpromazine, but the difference was not significant ($p<1, >0.05$) in spite of the use of large groups of rats. Whatever slight protection might have been exerted may, of course, be entirely due to the inhibition of the peripheral action of the adrenaline.

The foregoing experiments have not substantiated the hope, expressed by Courvoisier *et al.* (1953), that the administration of chlorpromazine might produce a kind of "chemical adrenalectomy" by inhibiting adrenomedullary secretion, or the claim by Aron *et al.* that the drug caused the equivalent of a hypophysectomy by preventing the release of ACTH. The explanation of the shock-preventing action of this drug, rather than lying in an inhibition of the adrenal defence mechanism of the organism, must be sought elsewhere —perhaps in the metabolic effects it produces.

Summary

1. Chlorpromazine (25 mg./kg. subcutaneously) does not inhibit the stimulation of the sympathetic centres produced by morphine in the cat, as judged by the following criteria: a fall in hypothalamic noradrenaline and a depletion of the stores of medullary amines in the innervated adrenal. During certain periods of the experiment, the amount of medullary amines released into the circulation dilates the (innervated and denervated) pupil and is thus sufficient to overcome the adrenolytic action of chlorpromazine. In contrast, nalorphine inhibits all manifestations of morphine poisoning including the stimulation of the sympathetic centres.

2. In the dog, the duration of the antiadrenaline effect of chlorpromazine was followed, and its

alleged inhibitory action on ganglionic transmission was examined ; no such action was found on the superior cervical ganglion.

3. The release of ACTH in operative shock was not prevented in the rat by chlorpromazine (10 mg./kg.) administered 3 hr. before the operation ; nor was the release of ACTH by adrenaline (200 μg./kg. subcutaneously) prevented. Chlorpromazine itself caused some release of ACTH at this dose level ; if the dose was increased to 15 mg./kg., the release of ACTH was of the same magnitude as that caused by the operation, or by an injection of adrenaline ; a satisfactory investigation of a possible inhibitory effect of such doses of chlorpromazine on the ACTH releasing power of other procedures was thus not possible.

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RELEASE OF HISTAMINE FROM SKELETAL MUSCLE BY SNAKE VENOMS

BY

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It has long been recognized that the physiological effects of an intravenous injection of snake venom bear much resemblance to the acute effects of histamine or anaphylaxis. The work of Gautrelet, Halpern and Corteggiani (1934, 1936) has demonstrated the similarity of the action of the venom of *Vipera aspis* and Indian cobra to that of histamine on the peripheral vascular system. Chopra and Chowhan (1934) and Chopra, Chowhan and De (1935) showed that *V. russellii* and *Echis carinata* caused death from acute circulatory failure. The viper venom produced hypotension and paralysis of capillaries resembling the effects of histamine or protein shock. The symptoms of vascular failure in *Echis* poisoning also resembled histamine shock. Feldberg and Kellaway (1937a) explained some of the effects of intravenous injections of cobra venom in cats and dogs in terms of histamine release, and demonstrated (1937b) that after injection of the venom into the pulmonary artery of the perfused guinea-pig's lungs the perfusate contained histamine. These authors (Feldberg and Kellaway, 1938) further showed that the addition of the venom to the fluid used for perfusing the liver or lung of the dog caused histamine to be liberated from these organs.

Several substances such as strychnine (Schild and Gregory, 1947), adrenaline (Eichler and Barfuss, 1940), curare alkaloids (Alam, Anrep, Barsoum, Talaat and Wieninger, 1939), and ammonia (Schild, 1949) are known to set free histamine from skeletal muscle. Since snake venoms liberate histamine from certain tissues, it was considered possible that they might have a similar action in skeletal muscle. The object of this work is to find out whether histamine is released from the rat diaphragm by snake poison and, if so, to compare quantitatively the rate of release of histamine by the venom of three well-known deadly-poisonous Indian snakes—cobra (*Naja naja*), krait (*Bungarus coeruleus*) and saw-scaled viper (*Echis carinata*). An account is also given of the effect of antihistamine drugs on the toxicity of snake venoms. A preliminary account of the release of histamine by cobra venom has already appeared (Dutta and Narayanan, 1952).

METHODS

The rat diaphragm preparation of Rocha e Silva and Schild (1949) was used. The diaphragm of the albino rat bred at this Institute, and weighing between 200 and 300 g., was dissected out from the surrounding structures. The two lateral lobes of the diaphragm, freed from the middle lobe, were used. One was placed in 5 ml. of oxygenated Tyrode solution at 37° C. and the other in Tyrode solution containing snake venom. These solutions were withdrawn and replaced at regular intervals by fresh solutions of the same composition. The active substances released by venoms were assayed biologically on isolated guinea-pig's ileum suspended in a small bath of Tyrode solution. The total volume of the solution in the bath was always adjusted to 4.5 ml. The active substances were compared with histamine acid phosphate. The activities are given in terms of histamine base.

At the end of the experiment, the histamine remaining in the muscle was first extracted by grinding the muscle with sand and acidified saline. The solution was boiled for a minute and centrifuged. The supernatant liquid was collected and the precipitate was washed with saline and centrifuged again. The process was repeated twice. The final volume of the supernatant fluid after neutralization was so adjusted that 10 ml. corresponded to 0.1 g. of the wet tissue.

RESULTS

Identification of the Substance Released by Venoms

Venoms of cobra, krait and saw-scaled viper released from rat diaphragm substances which produced rapid contraction of the guinea-pig's isolated ileum. When the bath was washed out, the gut relaxed as readily as after a dose of histamine. The contractions produced by the substances released from rat diaphragm, and by equiactive doses of histamine, were reduced to an equal degree by antazoline and tripelennamine, but were unaffected by atropine. This is illustrated in Fig. 1.

The substance released by cobra venom from rat diaphragm causes a depressor effect in the cat similar to that of histamine; this is unaffected by atropine.

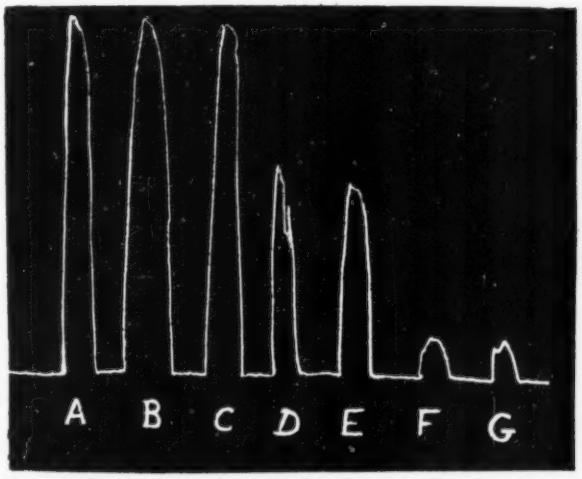

FIG. 1.—Contractions of guinea-pig's ileum in Tyrode solution: A, 0.03 μg. histamine; B, 0.1 ml. test solution containing the substance released by cobra venom (100 μg./ml.) from rat diaphragm; C, 0.1 ml. test solution in presence of 0..1 μg atropine; D, 0.1 ml. test solution in presence of 0.1 μg. antazoline; E, 0.03 μg. histamine in presence of 0.1 μg. antazoline; F, 0.1 ml. test solution in presence of 0.5 μg. antazoline; and G, 0.03 μg. histamine in presence of 0.5 μg. antazoline.

Since the test solution itself contained venom which is known to produce a fall of blood pressure in the cat, it was necessary to remove or destroy the venom before the true hypotensive action of the liberated substance could be demonstrated. This was achieved by using Code's (1937) method for the extraction of histamine. In a control experiment a known quantity of histamine was added to cobra venom solution; when this was treated by the above procedure, the depressor effect of the venom was completely removed whereas the added histamine was quantitatively recovered. Fig. 2 illustrates a typical experiment, and shows that the hypotensive property of the venom is removed by Code's method without the depressor response of the liberated agent being affected. Parallel quantitative assays of the histamine content of test solution on guinea-pig's ileum and on cat's blood pressure showed satisfactory agreement, within the error of the two methods. In a typical experiment, the histamine liberated from four rat diaphragms by cobra venom was pooled and concentrated. Direct assay of this solution on guinea-pig's ileum showed a histamine content equivalent to 21.1 μg./g. of tissue, whereas the same solution when assayed after Code's treatment gave a value of 20.3 μg./g. on guinea-pig's ileum and 22.3 μg./g. on cat's blood pressure. Further experiments showed that the active agent liberated from rat diaphragm by the venoms of saw-scaled viper and of krait was also indistinguishable from histamine.

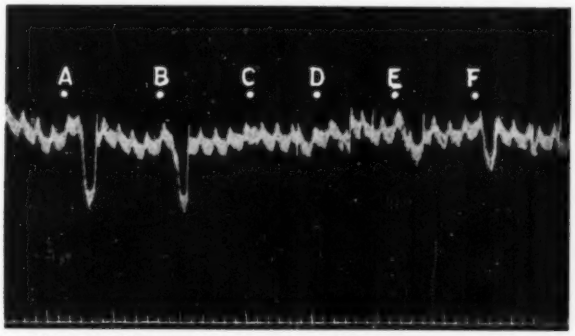

FIG. 2.—Cat, chloralose, atropinized. Blood pressure. A, histamine 0.17 μg.; B, 0.7 ml. test solution containing released substance and cobra venom, treated by Code's method; C, 0.7 ml. Tyrode solution having the same concentration of venom as in B but without the released agent, treated by Code's method; D, 1 mg. antazoline; E, 0.17 μg. histamine; and F, 0.7 ml. test solution. The recording was stopped between injections.

The Amount of Histamine Released by Venoms

Before the released histamine could be assayed on guinea-pig's ileum, the gut had to be desensitized to the venom, because all three venoms, in doses of 50 to 100 μg., produce contractions after a latent period of 6–10 sec. This is more pronounced with cobra venom than with the other two. On subsequent addition of similar doses of venom the contractions become less, and finally disappear. To desensitize the tissue against cobra venom about 100 to 200 μg. is required. Guinea-pig's ileum (Schild, 1939) is stated to contain appreciable amounts of histamine and these contractions may result from the liberation of tissue histamine by the venoms.

Fig. 3 shows the progressive decrease of the histamine content of the muscle when bathed repeatedly in Tyrode solution containing 100 μg./ml. of venom. The results for each venom are the average of seven experiments. Cobra venom seems the most active in liberating histamine and saw-scaled viper the least active.

Cobra venom set free 50–60% of tissue histamine in 10 min. and at the end of 45 min. the diaphragm was almost depleted of histamine. It will be observed that with other venoms the release of histamine was more gradual, so that at the end of 45 min. about one-fifth and one-third of the total histamine were retained in the muscle with the krait and the saw-scaled viper venoms respectively. Cobra venom in a concentration of 100 μg./ml. usually released as much as 40% of the total histamine in five min. as against 20% with the other two venoms.

There was a difference in the rate of release of histamine with larger and smaller doses (Table I). Lower concentrations of the venoms could release histamine only up to a certain limit, no matter how long the experiments were continued. For instance, 10 μg./ml. of cobra venom released only 57% of the total histamine from the muscle during the entire

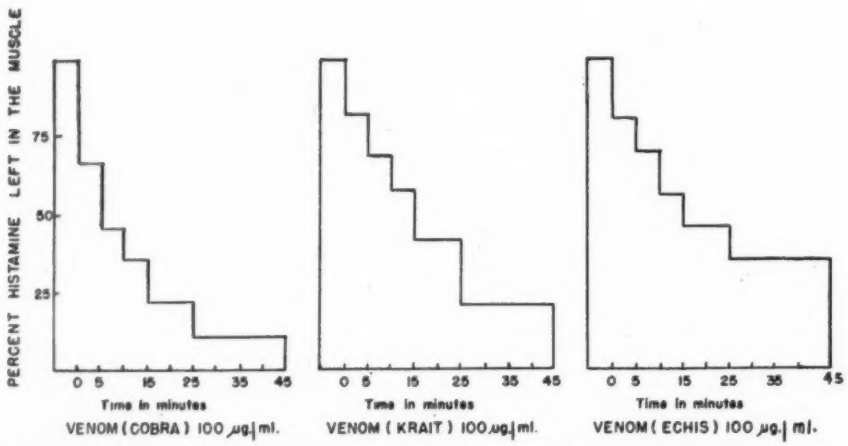

FIG. 3.—Comparison of histamine output from isolated rat diaphragm, in oxygenated Tyrode solution at 37° C., by the three venoms. Average of seven experiments with each venom.

TABLE I

RELEASE OF HISTAMINE FROM RAT DIAPHRAGM, IN OXYGENATED TYRODE SOLUTION AT 37° C., BY SNAKE VENOMS

(Histamine values are expressed as base)

Expt.	Release of Histamine (µg./g.)		Residual Histamine Content of Diaphragm (µg./g.)	Total Histamine Content of Diaphragm (µg./g.)
	During First 5 Min.	During 45 Min.		
	Cobra Venom, 10 µg./ml.			
1	2·80	11·25	6·30	17·55
2	2·16	9·10	8·31	17·41
3	3·50	11·95	5·45	17·40
4	2·60	5·76	8·90	14·66
5	3·20	7·33	7·09	14·22
6	2·42	13·41	8·32	21·73
7	1·69	5·96	3·85	9·81
Mean	2·62	9·25	6·90	16·15
	Cobra Venom, 50 µg./ml.			
1	4·90	14·25	3·80	18·05
2	4·97	11·47	4·30	15·77
3	3·90	13·28	3·20	16·48
4	6·70	20·54	2·10	22·65
5	2·90	7·97	6·20	14·17
6	5·32	14·32	1·80	15·12
7	4·14	12·14	2·72	14·86
Mean	4·69	13·42	3·49	16·91
	Echis Venom, 50 µg./ml.			
1	1·50	6·70	7·50	14·20
2	2·50	11·40	7·90	19·25
3	2·52	7·45	7·52	14·97
4	3·20	9·40	5·60	15·00
5	3·25	11·85	8·60	20·45
6	1·95	8·34	6·32	14·66
7	3·10	10·12	7·72	17·84
Mean	2·57	9·32	7·31	16·63
	Krait Venom, 50 µg./ml.			
1	1·90	11·75	5·50	17·25
2	2·50	17·60	7·80	25·4
3	1·75	6·58	3·80	10·38
4	3·25	13·60	7·0	20·6
5	1·28	8·53	3·25	11·78
6	2·63	10·51	6·18	16·69
7	1·62	12·47	4·76	17·23
Mean	2·13	11·57	5·47	17·04

experiment, but in the last five min. the histamine output was reduced to only 2.5 µg./g. of muscle. Thus, had the experiments been continued for more than 45 min. it is unlikely that any appreciable further quantities of histamine would have been released by the venom. Similarly, other experiments (not reported here) showed that higher concentrations (200 µg./ml.) of the venoms of krait and saw-scaled viper could, like cobra venom, deplete almost all the tissue histamine in 45 min.

Effects of Antihistamines on the Survival Period of Envenomed Animals

It has already been stated that clinical symptoms following snake bites often resemble the acute effects of histamine. Since snake poisons release histamine it seemed likely that at least some of these symptoms would be due to histamine release. The question arose whether antihistamines could prevent, or at least reduce, the effect of released histamine and thus afford some protection against the venom.

The results reported in Table II are based on a series of experiments in which the survival periods of envenomed animals treated with different antihistaminic drugs were compared with those of controls which had received venom but not antihistamines. For each experiment a group of 9 or 10 animals was selected, out of which 3 were used as controls. The groups consisted mostly of litter mates. All the animals in a group were injected on the same day and under identical conditions, the solutions of venom and antihistamine drugs being prepared fresh on the day of the experiment. The experiments were carried out on adult white rats (fasting weight 60–170 g.) and guinea-pigs (320–520 g.) bred and reared at this Institute. Cobra venom (0.5 mg./kg.) was injected intraperitoneally in the rat and intramuscularly in the guinea-pig. The treated animals received in addition two doses of an antihistamine drug intraperitoneally, the first dose being given 15 min. before, and the second 25 min. after, the administration of the venom.

Table II shows that the rats injected intraperitoneally with 0.5 mg./kg. cobra venom survived for a period of 65–175 min., and the guinea-pigs given the same amount of venom for 79–220 min. There is thus no difference between the survival periods of the two species of animals, although the routes of the injection of the venom were different. When the survival periods of envenomed animals

TABLE II

SURVIVAL TIMES OF ENVENOMED ANIMALS TREATED WITH ANTIHISTAMINES

(The antihistamines were given intraperitoneally 15 min. before and 25 min. after 0.5 mg./kg. cobra venom injected intraperitoneally to rats and intramuscularly to guinea-pigs.)

Expt. No.	No. of Animals	Mean Wt. (g.)	Antihistamine and Dose (mg./kg.)	Survival Time (Min.) Minimum and Maximum	Mean Survival Time Min.	Difference of Means ± S.E. Min.	Significance
			Rats				
1	3	160·0	Control	90–112	104·0	8·50 ± 8·46	t=1·005; p=0·175. Not significant
	6	157·7	Antazoline, 2·5	90–123	112·50		
2	3	140·0	Control	107–149	130·67	−26·67 ± 13·70	t=1·947; p=0·047. Significant
	6	123·0	Antazoline, 5	82–131	104·00		
3	3	140·0	Control	65–105	88·33	18·10 ± 13·14	t=1·377; p=0·105. Not significant
	7	125·0	Tripelennamine, 2·5	85–135	106·43		
4	3	138·6	Control	100–175	146·67	−10·84 ± 18·88	t=0·574; p=0·290. Not significant
	6	150·6	Promethazine, 2·5	115–150	135·83		
			Guinea-pigs				
5	3	433·3	Control	90–220	136·00	18·00 ± 33·03	t=0·545; p=0·300. Not significant
	6	387·5	Antazoline, 2·5	125–208	154·00		
6	3	506·6	Control	79–148	116·33	1·90 ± 15·32	t=0·124; p=0·460. Not significant
	7	480·7	Tripelennamine, 2·5	99–140	118·23		
7	3	400·0	Control	112–163	134·67	21·33 ± 24·92	t=0·856; p=0·240. Not significant
	6	378·0	Promethazine, 2·5	107–193	156·00		

treated with an antihistamine were compared with those of untreated animals, some prolongation of life of the treated animals was observed in a few experiments. For example, the control guinea-pigs in Expt. 5 (Table II) lived for an average period of 136 min. whereas the animals treated with 5 mg./kg. antazoline survived for 154 min. When the test was applied this difference was found to be statistically not significant (P=0.3). Likewise promethazine did not prolong significantly the life of guinea-pigs (Expt. 7) though the mean survival period of the treated animals was longer by 21.3 min. than that of the controls. In Expt. 2, on the other hand, with a larger dose of antazoline (10 mg./kg.) the mean survival period of the treated rats was 104 min., whereas that of the controls was 130 min. Here, the value of t is 1.95 (p=0.047), which is significant at the 5% level. The shorter span of life in the treated animals might possibly have been due to the toxicity of antazoline itself being added to the poisonous effect of the venom. Further experiments (Nos. 1, 3, 4 and 6) with tripelennamine and promethazine on rats and guinea-pigs did not give any indication that these drugs could significantly alter the survival periods when the animals were poisoned with cobra venom.

DISCUSSION

A "slow-reacting substance" which has some resemblance to the action of histamine is occasionally associated with substances capable of releasing histamine from animal tissues. Feldberg, Holden, and Kellaway (1938) observed it in lung perfusates following perfusion with snake venom. The existence in wasp venom of a potent unidentified substance which produces a delayed, slow contraction of guinea-pig ileum in the presence of atropine and mepyramine has recently been demonstrated by Jaques and Schachter (1954b). Other workers have observed the appearance of a slow-acting substance in the plasma of the cat when injected with 48/80 (Paton, 1951) or anemone extract (Jaques and Schachter, 1954a). In our experiments there was no evidence of the release of a similar slow-reacting substance from rat diaphragm, since the liberated agent was pharmacologically indistinguishable from histamine. The released substance, unlike the slow-reacting substance, produced a quick contraction of the guinea-pig ileum in the presence of

atropine and antihistamine; subsequent relaxation of the gut after washing was as rapid as after a dose of histamine. The histamine equivalent of a given sample was the same before and after treatment by Code's method.

Attempts to counteract the poisonous effect of cobra venom by antihistamines did not meet with success. There are several possible explanations for this failure:

(1) The antihistamine may not reach the site of action of intracellularly-released histamine. It has been pointed out by Feldberg and Paton (1951) that snake and bee venoms release histamine by enzymatic destruction of animal tissue. It is probable that under these conditions histamine is in fact liberated within the cell.

(2) Histamine may kill the animal by some action which is not counteracted by antihistamines. It is known that antihistamines are not very effective in neutralizing the depressor responses of histamine in the cat and dog (Dews and Graham, 1946; Marsh and Davis, 1947). It is also well known that the secretagogue action of histamine on the gastric mucosa is only partially antagonized by antihistamine drugs.

(3) Histamine release may not be the immediate cause of death by snake poison. In clinical cases histamine release probably accounts for the cold extremities, blanched pale skin, low blood pressure, rapid, thready pulse, and extreme prostration. However, the gross damage to the cellular tissues which snake venom produces may be the deciding factor of the fatal outcome, and the release of histamine may be incidental.

Summary

1. The venoms of cobra (*Naja naja*), krait (*Bungaras coeruleus*) and saw-scaled viper (*Echis carinata*) liberate histamine from the isolated rat diaphragm, cobra venom being the most active and saw-scaled viper venom the least active in this respect.

2. The release of histamine by cobra venom is explosive in character, as 50 to 60% of the histamine content of the muscle is set free during the first ten minutes.

3. Antihistamine drugs did not reduce the toxic effects of cobra venom on rats and guinea-pigs, the survival period being not significantly altered when envenomed animals were treated with antazoline, tripelennamine or promethazine.

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THE EFFECTS OF ANTIHISTAMINE SUBSTANCES ON THE RESPONSE TO HISTAMINE OF THE BLOOD VESSELS OF THE HUMAN FOREARM

BY

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Antihistamine drugs have been used by various workers (Emmelin and Emmelin, 1947; Folkow, Haeger and Kahlson, 1948) to investigate the dilator mechanisms in the peripheral circulation of animals. Since such mechanisms in man may conveniently be examined in the limbs (Landowne and Thompson, 1948), we have investigated the effects of antihistamine drugs introduced into the arterial circulation of a limb and observed their local action on the forearm vessels, their effect on the response of the forearm circulation to various doses of histamine, and the duration of this effect.

METHODS

The forearm blood flow was measured by venous occlusion plethysmography (Barcroft and Swan, 1953) with temperature-controlled plethysmographs at 34–35° C. (Greenfield, 1954). Four records of flow were usually made in each min. and the average of these taken as the flow in that min. and expressed in ml. of blood/100 ml. of tissue/min.

Intra-arterial infusions were given through a needle connected by polythene tubing to a mechanically driven syringe and inserted centrifugally, through an anaesthetized area of skin, into the brachial artery at the elbow. Two syringes could be driven simultaneously and the infused solution thus changed without delay. An infusion of physiological saline was maintained at a rate of 4 ml./min. except when the drug solutions, made up in physiological saline so that the dose for 1 min. was contained in 4 ml., were being given. In some expts. infusions were given into an antecubital vein.

The antihistamines were: tripelennamine hydrochloride ("Pyribenzamine"), antazoline ("Antistin"), and mepyramine maleate ("Anthisan"). The amount infused into the brachial artery was usually half the intravenous dose recommended by the manufacturers and was usually given over a period of 5 min.

Histamine acid phosphate (1:1,000, B.D.H.) was given intra-arterially in doses of 1, 5, 10, or 20 μg./min. for periods of either 3 or 5 min. Doses are expressed as the weight of the salt.

The room temperature was 20–22° C. and the subjects (colleagues and medical students) were recumbent for at least half an hour before observations were begun.

RESULTS

General Effects of Antihistamines

In a total of 26 expts., in which 12.5 mg. of tripelennamine, 50–100 mg. of antazoline, or 25 mg. of mepyramine were given into the brachial artery, no general effects were experienced by the subjects either during or after the infusion.

Intravenous infusion of these doses of tripelennamine and antazoline usually had no general effects. In one subject, however, 25 mg. of tripelennamine intravenously caused, during the infusion, sensations of dizziness, slight nausea and tingling in the limbs. These symptoms passed off during the subsequent 45 min. Similar symptoms occurred in one subject after 25 mg. of mepyramine; the effects were more persistent than with tripelennamine and the drowsiness lasted for 5 hr.

Local Effects of Antihistamines

Tripelennamine (2.5 mg./min. for 5 min. in 9 expts.) infused intra-arterially caused a fall in blood flow during the infusion to about half the resting value, but antazoline (10 mg./min. for 5 min. in 9 expts.) and mepyramine (5 mg./min. for 5 min. in 3 expts.) caused a twofold increase in flow. After tripelennamine there was a further slight fall and the flow usually remained at this lower value throughout the remainder of the expt. (Fig. 1). After antazoline and mepyramine, however, the blood flow gradually fell to, or a little below, the previous resting level when the infusion ceased.

When the intra-arterial infusion of antazoline (5 expts.) or mepyramine (1 expt.) was repeated, up to 34 min. later, the dilator effect was not reduced.

No effect on forearm blood flow was detected during intravenous infusion of any of the antihista-

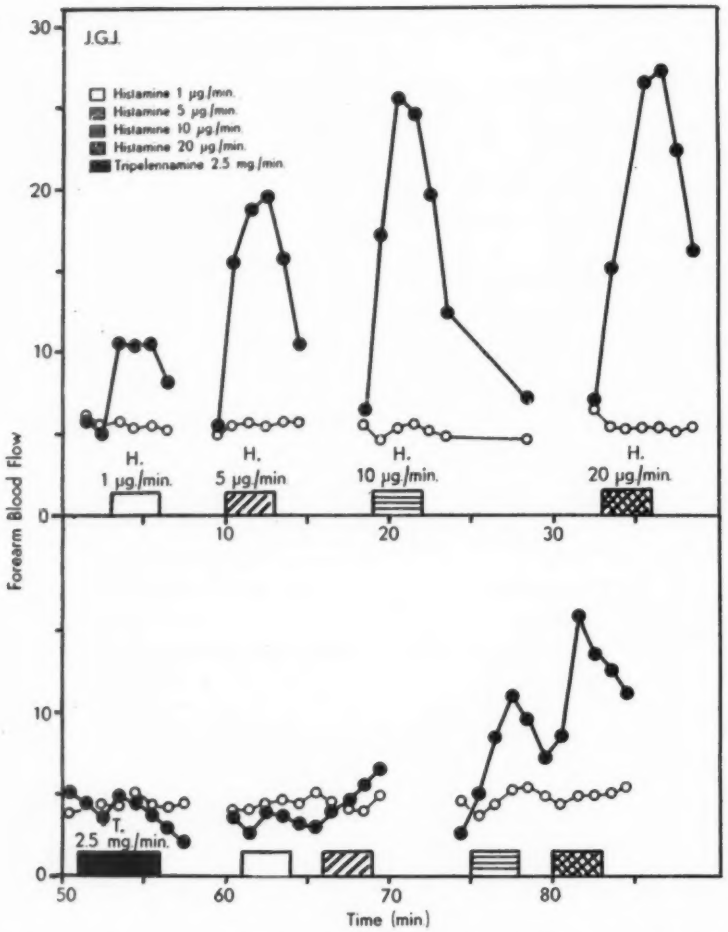

FIG. 1.—The response in one subject of the forearm circulation to four different doses of histamine infused into the brachial artery before and after infusion of tripelennamine. The forearm blood flow is expressed in ml./100 ml./min. and each symbol represents the average of four observations of flow taken in 1 min. The closed circles indicate the flow in the injected forearm and the open circles that in the opposite forearm.

mine substances (5 expts.). In one of these expts. both the hand and the forearm blood flows were measured while antazoline was given intravenously in successive doses of 1, 2, 5 and 10 mg./min. each for 5 min. There was no alteration in the flow.

In 1 expt. in which the skin was observed during the intra-arterial infusion of 50 mg. antazoline in 5 min. there was a slight flush of the forearm skin and there was a feeling of distension and tingling in the arm. A second infusion of the same dose 18 min. later had no such effects. This was the only occasion on which symptoms were noticed in the forearm with any of the antihistamines. Usually the effect of the antihistamines on the skin colour was not observed because of the plethysmograph on the forearm.

Effect of Intra-arterial Antihistamines on Histamine Response of Forearm Vessels

This was determined in the following way. The blood flow was measured throughout in both forearms. The vasodilator response of one forearm was first observed to doses of 1, 5, 10, and 20 μg./min. of histamine given in that order into one brachial artery. The flow on the injected side was allowed to return to the base-line value between the doses of histamine. The antihistamine substance was then introduced into the brachial artery, the flow being measured continuously. Histamine infusions were then repeated in the same doses and order as before. Finally, at the end of the expt. to determine whether the effectiveness of the antihistamine had diminished, a further dose of 1 μg./min. of histamine was given and the response compared with that immediately following the antihistamine.

Fig. 1 shows the results of a typical experiment with tripelennamine.

Tripelennamine, in a total dose of 12.5 mg., reduced the responses of the forearm circulation to all four doses of histamine (Fig. 2A) and was still effective at the end of the series. In A.H.G.L. the response to 1 μg./min. of histamine was abolished; the responses to 5 and 10 μg./min. were less than, and that to 20 μg./min. little more than, the response to 1 μg./min. before the antihistamine had been given. The effect in J.G.J. was similar. Some further results with tripelennamine are shown in Fig. 3A.

Mepyramine was less effective than tripelennamine as an antihistamine (Fig. 2), and there was some recovery from its antihistamine effect by the end of the expt.

Antazoline was almost as effective as mepyramine in reducing the dilator responses to 1 μg./min. of histamine, but the effect on the responses to the higher doses was less marked (Fig. 2C). This was probably due to the wearing off of the antihistamine effect by the time the higher doses were given, because at the end of the series (¾–1 hr. after the injection of antihistamine) the response to 1 μg./min. of histamine had almost recovered in P.J.G. and was greater than before

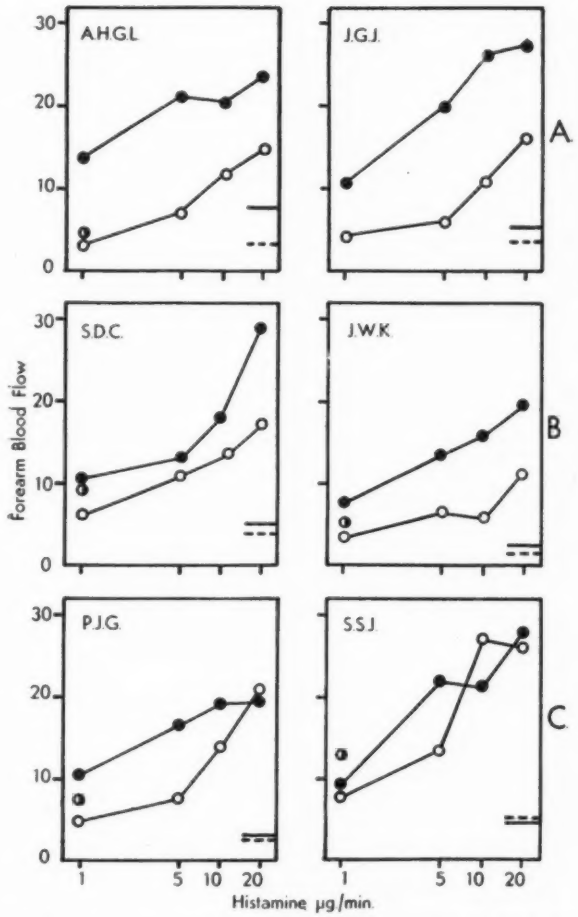

FIG. 2.—The response of the forearm circulation to histamine before and after intra-arterial infusion of A, tripelennamine (12·5 mg.), B, mepyramine (25 mg.), and C, antazoline (50 mg.). The forearm flow is expressed in ml./100 ml./min. and the abscissa represents the dose of histamine in μg./min. on a log. scale. The symbols represent the average of the highest four flows during the 3 min. periods of histamine infusion. ● before antihistamine; ○ 15 min. after antihistamine; ◑ 1–1¼ hr. after antihistamine; —— resting level of flow before antihistamine; - - - - resting level of flow after antihistamine.

in S.S.J. The additional data shown in Fig. 3B confirm that antazoline is less effective than tripelennamine.

Effect of Repetition of Antihistamines.—In 5 expts. an attempt was made to increase the effectiveness of antazoline by giving a second dose after the first had been tested by histamine infusions. Fig. 4 shows the results on one of the subjects. The inhibiting effect of the first dose of antazoline was not very satisfactory, but a second dose did not further inhibit the effect of 5 μg./min. of histamine. The data for all 5 expts. with antazoline, and for 1 with mepyramine, are shown in Table I. The effectiveness of an antihistamine was never increased by a second dose.

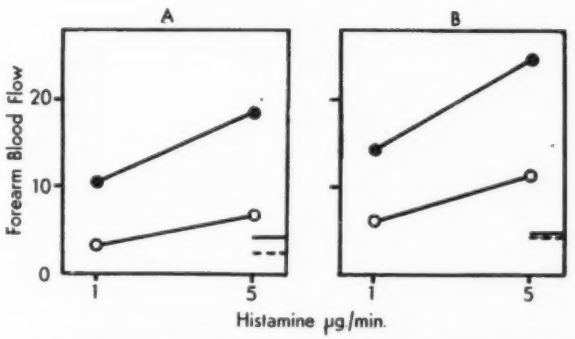

FIG. 3.—Averaged data from expts. in which A, tripelennamine (8 expts.), and B, antazoline (6 expts.), were used in investigating histamine release in the forearm. The symbols represent the highest four flows during infusion of histamine in doses of 1 and 5 μg./min. before (●) and after (○) antihistamine. The forearm flow is expressed in ml./100 ml./min.; —— resting level of flow before antihistamine; - - - - resting level of flow after antihistamine.

Effect of Intravenous Antihistamines on Histamine Response of Forearm Vessels

The effect of intravenous administration of tripelennamine (12.5 mg. in 5 min.) on the response of the forearm circulation to intra-arterial histamine was investigated in one subject (Fig. 5A). This dose of antihistamine given in this way is less effective and less persistent than is the same dose given intra-arterially (Fig. 2A). One hour after administration of the antihistamine the responses had returned to normal.

Effect of Intra-arterial Antihistamine on Histamine Response of the Opposite Forearm

The validity of the opposite forearm as a control in expts. in which antihistamine is given intra-arterially depends upon whether or not sufficient antihistamine enters the general circulation to affect the histamine response of the opposite forearm.

TABLE I

EFFECT OF REPEATED DOSES OF ANTIHISTAMINE ON THE FOREARM RESPONSE TO HISTAMINE

Subject and Rate of Histamine Infusion		Average of Highest 4 Flows During Histamine (ml./100 ml./min.)		
		Before	After 1st Dose	After 2nd Dose
		Antazoline		
A.D.M.G.	1 μg./min.	14·6	6·2 (50 mg.)	8·1 (50 mg.)
	5 μg./min.	25·8	14·7	14·7
S.P.D.	5 μg./min.	16·6	12·4 (100 mg.)	13·7 (100 mg.)
I.V.A.	1 μg./min.	12·2	6·1 (50 mg.)	6·2 (50 mg.)
J.J.	5 μg./min.	13·2	9·6 (20 mg.)	8·9 (20 mg.)
R.F.W.	4 μg./min.	22·0	12·1 (40 mg.)	11·3 (60 mg.)
	0·75 μg./min.	—	5·9	5·9
		Mepyramine		
J.R.	1 μg./min.	6·9	4·0 (25 mg.)	3·8 (25 mg.)
	5 μg./min.	10·2	5·8	6·1

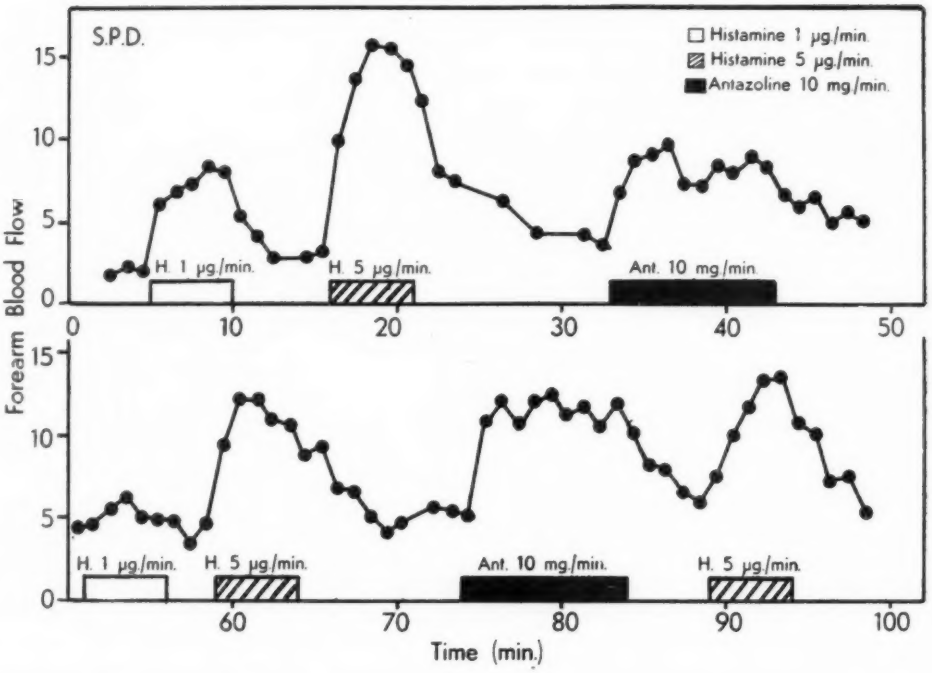

FIG. 4.—The response of the forearm circulation to 5 min. infusions of histamine before and after intra-arterial infusions of antazoline. The conventions are the same as in Fig. 1. Two doses of antazoline were given, each of 100 mg. over 10 min. The flow through the injected forearm only is shown.

Fig. 5B shows an expt. in which the effects of injecting histamine at 1 and 5 μg./min. into the left brachial artery were observed before and after injecting 12.5 mg. of tripelennamine in 5 min. into the contralateral brachial artery. The response to histamine was conspicuously reduced, but less so than after injection of tripelennamine into the ipsilateral brachial artery on another occasion. The responses to both 1 and 5 μg./min. of histamine, in terms of the maximum increase above the previous resting level, were reduced to about two-thirds of those before the antihistamine, whereas in all 9 expts. in which tripelennamine was infused intra-arterially, and the histamine responses measured in the forearm of the same side, the responses to 1 μg./min. were almost abolished and those to 5 μg./min. reduced to less than one third.

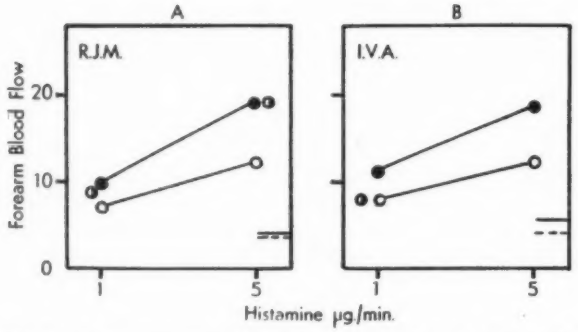

FIG. 5.—A, The response of the forearm circulation to intra-arterial histamine following intravenous infusion of 12·5 mg. of tripelennamine. B, The response of the circulation through the left forearm following infusion of 12.5 mg. of tripelennamine into the right brachial artery. The conventions are the same as in Fig. 2.

Intra-arterial Antihistamines on the Response to Electrophoresis of Histamine into Skin

In one subject histamine was administered by electrophoresis into the flexor surface of the forearm before and after the infusion of 50 mg. of mepyramine into the brachial artery. Before the antihistamine was given this procedure resulted in, first, a redness of the circular area in contact with the histamine solution, then an irregular flare, and, finally, a circular weal over the central area. Sixty min. after the mepyramine had been given, and shown to be effective in reducing the responses of the forearm blood flow to intra-arterial doses of 1 and 5 μg./min. of histamine, the procedure was repeated. The circular area of redness was obtained as before, but neither the flare nor the weal developed.

DISCUSSION

The antihistamine substances tripelennamine, antazoline and mepyramine when introduced intra-arterially into the forearm, in half the intravenous dose recommended by the makers, are effective in reducing or abolishing the dilator response of the forearm circulation to histamine introduced by the same route. Tripelennamine and

mepyramine were more satisfactory than antazoline and their effect persisted for at least 1–1½ hr. whereas antazoline had usually ceased to be effective at the end of this time. It was not possible in a single expt. to assay the effect of the antihistamine on the response of the forearm flow to various doses of histamine given in different sequences because of the length of time required for one sequence, largely taken up in waiting for the blood flow through the forearm to return to the resting value between histamine infusions. Recording the responses to 4 doses of histamine before and after an infusion of antihistamine occupied about 3 hr., and this was as long as even a trained subject was content to lie with both arms supinated and immobilized in plethysmographs and with a needle in one brachial artery. With tripelennamine the weaker effect on the responses to the larger doses of histamine was not due to the wearing off of the antihistamine effect, because when 1 μg./min. of histamine was repeated at the end of the series the response was still almost or completely abolished. With antazoline, however, the absence of any reduction of the responses to the higher doses was almost certainly due to the short duration of the effect of the drug as shown by the presence of a normal response to 1 μg./min. of histamine at the end of the series. With mepyramine the responses to the higher doses showed considerable reduction, but there was some recovery by the end of the expt.

Antazoline and mepyramine both had a sustained dilator effect on the forearm vessels while they were being infused into the brachial artery, and this effect recurred on repetition of the dose. Thus, although it has been shown that antihistamines may act as histamine releasers (Pellerat and Murat, 1946 ; Arunlakshana, 1953) it appears that the increase in flow produced by the antazoline and mepyramine is unlikely to result from the release of histamine at a site accessible to the antihistamine action of the drugs.

Repetition of the antihistamine did not increase its effectiveness in reducing the response to histamine. Whether the antihistamine effect might have been enhanced by further increasing the dose of the drug is not known, since, in order to avoid undesirable side effects, amounts of more than 200 mg. have not been given. Folkow *et al.* (1948) found in the cat that increasing the dose of diphenhydramine above a certain value did not increase the antihistamine effect.

Histamine in a dose of 1 μg./min. causes an increase in forearm flow to about 10 ml./100 ml./min. and 5 μg./min. increases it to about 20 ml./100 ml./min. The ability of the intra-arterial antihistamine substances to reduce or abolish these responses suggests that the antihistamines could be used in the investigation of dilator phenomena in the forearm. The intra-arterial administration has the advantages that it produces a high local concentration, and that the dose of antihistamine can be kept below that which induces unpleasant or undesirable side effects. The most effective reduction of histamine responses was achieved with tripelennamine, and its duration of effect of at least 1–1½ hr. would enable observations of most vasodilator phenomena to be made while the drug was still effective. The individual variation in degree and duration of effect of the antihistamines makes it desirable that in any such investigation a check be made on the effectiveness of the drug in inhibiting histamine responses before and after the observations on the vasodilator phenomenon.

The value of the antihistamine introduced intra-arterially in the investigation of the possible rôle of histamine in vasodilator phenomena of the forearm depends upon whether the antihistamine so introduced is as effective in counteracting the response to histamine released in the tissues as it is in counteracting that introduced into the artery. Since the triple response caused by electrophoresis of histamine into the skin is abolished by antihistamine introduced intra-arterially, it seems likely that the antihistamine would reach the site of action of released histamine on the vessels, but it is possible that the histamine released from the tissues is in a form not amenable to counteraction by antihistamine. Our expts. provide no evidence on this point.

The fact that the introduction of tripelennamine into the brachial artery of one side resulted in a reduction of the response to histamine of the opposite forearm circulation indicates that, although the antihistamine was not entering the general circulation in sufficient quantities to cause any symptoms, enough was arriving in the forearm to reduce its response to histamine. The extent of this reduction (Fig. 5B) was approximately as great as that produced by the same dose given intravenously (Fig. 5A). Although only 2 expts. of this sort have been carried out, and individual variations in the response of the forearm both to histamine and to antihistamine might account for this similarity, it suggests that a considerable proportion of the intra-arterially introduced antihistamine enters the general circulation and reaches the opposite forearm. This implies that, in the investigation of the possible rôle of histamine in the cause of a vasodilator phenomenon

of the forearm, the opposite forearm cannot be used as a control, since the response to any histamine present or released there will be modified in the same way as, though to a lesser extent than, on the experimental side.

Summary

1. The effectiveness of intra-arterial tripelennamine, mepyramine and antazoline in inhibiting the response of the forearm circulation to histamine has been investigated.

2. Half the recommended intravenous dose of each drug abolishes, or almost abolishes, the response to 1 μg./min. of histamine given intra-arterially and very considerably reduces the responses to higher doses.

3. The greatest and most prolonged effect was from tripelennamine and the least and shortest effect from antazoline.

4. Repeating the dose of mepyramine and antazoline did not increase the antihistamine effect.

5. The intra-arterial administration of antihistamines in the investigation of the rôle of histamine in vasodilator phenomena of the forearm is discussed.

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FURTHER OBSERVATIONS ON THE EFFECT OF AMINOPTERIN, A-METHOPTERIN AND *CITROVORUM* FACTOR ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

BY

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A number of workers have reported on the effect of aminopterin and A-methopterin on the course of the growth of the Rous sarcoma and certain transmissible tumours in chicks. Woll (1948) and Little, Sampath, Paganelli, Locke and Subbarow (1948) have shown that a number of folic acid antagonists completely or partly inhibit the growth of the Rous chicken sarcoma although some toxic effects were encountered. The latter workers showed that the toxicity of aminopterin, in particular, could be partially neutralized by either pteroyldi- or pteroyltriglutamic acid in a 25 to 1 ratio, but such mixture showed no virus-inhibiting effect. Stock, Biesele, Burchenal, Karnofsky, Moore and Sugiura (1950) have also demonstrated that several analogues of folic acid exert an adverse effect upon tumour tissue. Ringsted (1952) fed a balanced chick mash containing aminopterin (5 mg./kg. of food) and found that the survival rate of chicks inoculated with Rous sarcoma was significantly higher at the end of the first week compared with that of chicks receiving no drug. The mean time of first appearance of the tumour in the survivors was also greater in the chicks receiving aminopterin, but the tumour incidence in both the medicated and control groups was 100%. Bessis and Freixa (1950) found that aminopterin and A-methopterin offered partial protection against a transmissible erythroblastosis in young chicks, but some intoxication was encountered. Ringsted (1953) showed that aminopterin in a chick mash at levels of 5, 10, 20 and 40 mg./kg. increased the mean induction time of a Mill Hill endothelioma in young chicks. The two higher levels proved toxic, and the drug at all levels had no effect on the total number of takes and subsequent growth-rate of the tumours. Darcel (1953a) failed to show any increase in the time taken for tumours to appear when aminopterin or A-methopterin were given orally to chicks bearing tumour strain RPL 12. The drugs were administered directly into the crop in aqueous suspensions (0.1 and 1.0 mg. of each drug/day). No toxic symptoms were reported.

Recently, *citrovorum* factor, a substance closely related to folic acid, has been found to be many times more effective than folic acid itself in reversing the toxicity of folic acid antagonists in the rat, chick, and man, and in this paper a number of observations are presented on the effect of aminopterin, A-methopterin and synthetic *citrovorum* factor on the growth of transplantable lymphoid tumour strains isolated from cases of avian leukosis.

METHODS

The general characteristics of the tumour strains used in the present study, RPL 12 and 19, have been described by Darcel (1953b). A tumour develops at the site of inoculation followed by rapid metastasis, in the absence of leukaemia, to the viscera, especially the liver.

Crossbred (B.R. × Br.L. and B.R. × W.L.) chicks were inoculated with tumour cell suspensions either at two or seven days of age. The birds were maintained in small metal cages and fed freely on a balanced chick mash.

The tumour cell suspensions were prepared in physiological saline with a special mincer (Craigie, 1949) from liver in which tumours were growing (artificially inoculated). The tumour suspension (0.25 ml.) was inoculated subcutaneously above the pectoral muscle. Concentrations of the tumour mince corresponded to 1/10th of the original tumour in all experiments. The chicks were palpated daily in order to calculate the mean time of first appearance of tumours in individual groups. The mean survival times of the experimental groups were also determined.

The drugs were administered either intraperitoneally or in the food. The *citrovorum* factor (20 μg.) was given intraperitoneally each day.

TABLE I

THE EFFECT OF INTRAPERITONEAL ADMINISTRATION OF AMINOPTERIN AND *CITROVORUM* FACTOR ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

Expt. No.	Tumour	Chicks Used	Age (Days)	No. Inoculated	Amount of Aminopterin Administered (μg. Daily)	*Citrovorum* Factor (μg. Daily)	Tumours		Deaths	
							Tumours Palpated	Mean Time of First Appearance (Days) ± Mean Deviation	No.	Survival Time (Days) ± Mean Deviation
1	RPL 19	BR/Br.L × BR	2	10	Nil	Nil	4	5·00±0	10	6·40±0·92
	,,	,,	2	10	100	,,	0	—	10	3·80±1·20
	,,	,,	2	10	100	20	5	6·80±1·04	9	7·44±1·16
2	RPL 12	BR × Br.L	2	10	Nil	Nil	10	7·80±0·80	10	13·20±1·04
	,,	,,	2	10	50	,,	0	—	10	3·60±0·72
	,,	,,	2	10	50	20	8	7·88±0·88	9	11·50±0·50
4	RPL 19	BR × W.L.	7	10	Nil	Nil	10	6·40±0·48	7	*10·00±0·56
	,,	,,	7	10	50	,,	0	—	10	7·50±2·60

* Three chicks were killed on the 25th day after inoculation and in each only a small regressing pectoral tumour was found.

RESULTS

In general, both aminopterin and A-methopterin were found to be toxic for young chickens, although a considerable variation in susceptibility was encountered. Aminopterin, in particular, proved extremely toxic when administered intraperitoneally to two-day-old chicks in daily doses of 50 or 100 μg. All the birds died within four days of the commencement of therapy and it was impossible to ascertain if any tumour-inhibiting action had occurred (Table I). These birds rapidly lost weight, anaemia and leucopenia were observed, and on post-mortem examination the liver was slightly enlarged and intensely yellow—probably the result of an accumulation of unmetabolized aminopterin. *Citrovorum* factor gave full protection against this toxicity when administered intraperitoneally at 20 μg./day. On the other hand, any possible tumour-inhibiting effect by the aminopterin was neutralized, there being no significant difference between the number of tumours palpated, the mean times of their first appearance, or mean survival times in either the treated or control groups (Table I, Expts. 1 and 2). The administration of aminopterin intraperitoneally to older chicks (50 μg. daily) whilst apparently preventing the appearance of palpable tumours ultimately caused a 100% mortality from intoxication (Table I, Expt. 4). The same picture was seen when aminopterin was administered in the diet (10 mg./kg. of food), although in week-old chicks it appeared to be less toxic (Table II, Expts. 4 and 5). No palpable tumours could be detected in these birds, with the exception of one in Expt. 5, and their mean survival time was significantly prolonged. With the exception of three survivors

TABLE II

THE EFFECT OF THE DIETARY ADMINISTRATION OF AMINOPTERIN ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

Expt. No.	Tumour	Chicks Used	Age (Days)	No. Inoculated	Dietary Level of Aminopterin (mg./kg.)	Tumours		Deaths	
						Tumours Palpated	Mean Time of First Appearance (Days)	No.	Survival Time (Days)
3	RPL 19	BR × W.L.	2	10	Nil	10	6·00±0	10	7·90±0·18
	,,	,,	2	10	10	0	—	10	5·10±1·14
4	RPL 19	BR × W.L.	7	10	Nil	10	6·40±0·48	10	10·00±0·56
	,,	,,	7	10	10	0	—	10	13·56±2·05[1]
5	RPL 12	BR × Br.L.	7	10	Nil	8	6·00±0	8	11·00±0[2]
	,,	,,	7	10	10	1	10·00±0	10	24·50±3·90[3]
6	RPL 19	BR × Br.L.	7	10	Nil	10	6·70±0·42	9	13·56±3·65
	,,	,,	7	10	10	0	—	10	3·43±0·65[4]

NOTES.—[1] One chick died from intoxication after 4 days and has not been included in the calculation of the mean survival time.
[2] Two chicks were killed after 35 days and no evidence of tumours was found.
[3] Four chicks died from intoxication after 4.9 days and have not been included in the calculation of the mean survival time.
[4] Three chicks survived to the 13th day and no tumours were observed.

TABLE III
THE EFFECT OF INTRAPERITONEAL ADMINISTRATION OF A-METHOPTERIN AND *CITROVORUM* FACTOR ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

Expt. No.	Tumour	Chicks Used	Age (Days)	No. Inoculated	Amount of A-methopterin Administered (μg. Daily)	*Citrovorum* Factor (μg. Daily)	Tumours		Deaths	
							Tumours Palpated	Mean Time of First Appearance (Days)	No.	Survival Time (Days)
1	RPL 19	BR × BR.L × BR	2	10	Nil	Nil	4	5·00±0	10	6·40±0·92
	,,	,,	2	10	100	,,	0	—	10	7·90±1·16
	,,	,,	2	10	100	20	8	5·00±0	10	6·10±0·72
2	RPL 12	BR × BR.L	2	10	Nil	Nil	10	7·80±0·80	10	13·20±1·04
	,,	,,	2	10	100	,,	0	—	10	*3·56±0·70
	,,	,,	2	10	100	20	10	7·00±0	10	11·10±0·36
3	RPL 19	BR × W.L.	2	10	Nil	Nil	10	6·00±0	10	7·90±0·18
	,,	,,	2	10	50	,,	4	7·75±0·38	10	10·00±0·40
4	RPL 19	7BR × W.L.	7	10	Nil	Nil	10	6·40±0·48	7	10·00±0·56
	,,	,,	7	10	50	,,	0	—	10	7·70±3·44

* One chick survived for 14 days before dying from liver lesions. This was not included in the calculations of the mean survival time.

in Expt. 6, all the birds eventually succumbed and were found to have diffuse lesions in the liver as the result of a lymphocytic infiltration.

A-methopterin, in general, proved somewhat less toxic than aminopterin, but once again considerable variation in susceptibility was encountered. In birds which were resistant to intoxication, both the intraperitoneal and dietary administration of the drug considerably reduced the number of palpable tumours and increased their time of first appearance (Tables III and IV). It was found, however, that A-methopterin in the diet at 10 mg./kg. had no effect on the number of tumours palpated, the mean time of their first appearance or survival time in two-day-old birds bearing RPL 19 (Table IV, Expt. 3). When the dose of the drug was raised to 20 mg./kg. and given to slightly older birds there was a marked reduction in the numbers of tumours palpated and an increase in survival time. In a single expt. with RPL 12, A-methopterin at 10 mg./kg. of diet showed some tumour-inhibiting effect (Table IV, Expt. 5). As with aminopterin, full protection against any toxicity was given by *citrovorum* factor (20 μg. daily intraperitoneally), but any tumour-inhibiting action was neutralized.

DISCUSSION

From the results presented, there is some evidence that both aminopterin and A-methopterin can influence the growth of transplantable avian lymphoid tumours. The results, however, are complicated by the toxicity of these two folic acid antagonists, although a wide variation in susceptibility has been encountered. Bennette (1952), in experiments with A-methopterin in mice, concluded that the association between the toxicity and tumour growth inhibition was so close that no confidence could be placed in the selectivity of the drug against certain sarcomata. Our results, in

TABLE IV
THE EFFECT OF THE DIETARY ADMINISTRATION OF A-METHOPTERIN ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

Expt. No.	Tumour	Chicks Used	Age (Days)	No. Inoculated	Dietary Level of A-methopterin (mg./kg.)	Tumours		Death	
						Tumours Palpated	Mean Time of First Appearance (Days)	No.	Survival Time (Days)
3	RPL 19	BR × W.L.	2	10	Nil	10	6·00±0	10	7·90±0·18
	,,	,,	2	10	10	10	6·00±0	10	8·20±0·36
4	RPL 19	BR × W.L.	7	10	Nil	10	6·40±0·48	10	10·00±0·56
	,,	,,	7	10	20	0	—	9	13·89±1·46
5	RPL 12	BR × Br.L.	7	10	Nil	8	6·00±0	8	11·00±0[1]
	,,	,,	7	10	10	2	8·00±1·00	9	13·83±3·44[2]
6	RPL 19	BR × Br.L.	7	10	Nil	10	6·70±0·42	9	13·56±3·63
	,,	,,	7	10	20	0	—	10	15·50±1·33[3]

NOTES.—[1] Two chicks were killed after 35 days and no evidence of tumours was found.
[2] Three chicks died from intoxication after 4.7 days and have not been included in the calculation of the mean survival time.
[3] Four chicks died from intoxication after 4.9 days and have not been included in the calculation of the mean survival time.

part, tend to confirm this conclusion—especially when the drugs are administered intraperitoneally to very young chicks. A greater resistance to intoxication was found when the drugs were administered in the food to week-old birds. In such resistant birds there was a considerable reduction in the number of palpable tumours and an increase in the time taken for them to appear, irrespective of the tumour strain used. In addition, the survival time was increased. However, when death intervened a post-mortem examination revealed that in the majority (even in the absence of a pectoral tumour) metastasis to the viscera had occurred, and especially to the liver.

The toxic effects of these two drugs could be overcome by injections of synthetic *citrovorum* factor, as has recently been demonstrated in the rat and chick (Sauberlich, 1953 ; Sauberlich and Schaefer, 1954). At the same time, this *citrovorum* factor blocked any tumour-inhibiting action. Because of the small amount of *citrovorum* factor available it was not possible to determine the minimum concentration which could prevent this intoxication, and yet permit the drugs to exert any possible tumour-inhibiting action. For the same reason, it was not possible to investigate the effect of its oral administration.

Darcel (1953a) found that the oral administration of suspensions of either aminopterin or A-methopterin failed to influence the growth of tumour strain RPL 12 and he found no evidence of intoxication. This suggests that little or no absorption of either drug had occurred. The toxic effects encountered in our experiments were poor growth, anaemia and leucopenia, and death. Bessis and Freixa (1950) administered the two antagonists intramuscularly in much higher doses (0.5 mg. of aminopterin and 4 mg. of A-methopterin) and reported only a small number of chicks showing intoxication. This route of administration may have permitted the very slow dispersion of the drug from a high local concentration, so that the tumour-inhibiting action was exerted without drastic interference with folic acid or *citrovorum* factor enzyme systems.

The above results suggest that the effect of these drugs on the growth of lymphoid tumours should be extended, especially as their toxic effect may be controlled by *citrovorum* factor. On the other hand, although there is some evidence that inhibition of tumour growth occurs in those chicks resistant to intoxication, death ultimately ensues from a diffuse lymphocytic infiltration of the viscera.

Summary

1. Aminopterin (4-aminopteroylglutamic acid) given intraperitoneally to chicks, bearing either tumour strain RPL 12 or RPL 19, in doses of 50 or 100 μg./day was highly toxic, and resulted in poor growth, anaemia, leucopenia and death. For this reason it was not possible to ascertain if any tumour inhibition had occurred.

This toxicity could be counteracted by *citrovorum* factor administered intraperitoneally, but any tumour-inhibiting action was neutralized.

2. Aminopterin (10 mg./kg. of diet) was somewhat toxic to chicks ; but, in those which were resistant, there was a considerable reduction in palpable tumours and an increase in survival time.

3. A-methopterin (4-amino-10-methylpteroylglutamic acid) given intraperitoneally to chicks, bearing either tumour strain RPL 12 or RPL 19, in doses of 50 or 100 μg./day was less toxic than aminopterin and exhibited some tumour-inhibiting action. The toxicity was completely counteracted by *citrovorum* factor administered intraperitoneally, but the tumour-inhibiting action was neutralized.

4. A-methopterin (10 mg./kg. of diet) was somewhat toxic, but in resistant chicks bearing RPL 12 there was a reduction in the number of palpable tumours and an increase in survival time. In chicks bearing RPL 19 this level was ineffective, but on raising it to 20 mg./kg. of diet a tumour-inhibiting effect was found.

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EFFECT OF DENERVATION ON ENZYMES IN IRIS AND BLOOD VESSELS

BY

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An investigation was described in 1952 by Burn and Robinson in which the effect of denervation on the amine oxidase present in the nictitating membrane, the iris and the blood vessels of the foreleg of the cat was studied. Denervation of the nictitating membrane and of the iris consisted in removal of the superior cervical ganglion and denervation of the foreleg arteries in removal of the stellate ganglion. The number of observations made on the nictitating membrane was large; these showed that in the period 9–12 days after removal of the ganglion the amine oxidase present diminished to about 60% of that on the normal side, but that during the next three weeks the amount increased once more until it was about the same as on the normal side.

The recovery of amine oxidase suggested that re-entry of sympathetic fibres into the nictitating membrane had taken place, most probably from fibres originating in the stellate ganglion and passing along the vertebral arteries. Burn and Robinson (1953) therefore carried out a second investigation in which both the superior cervical and the stellate ganglia were removed; they observed a fall in amine oxidase to a mean value of 50% of the normal, which persisted; the amount of amine oxidase did not recover as in the earlier experiments.

The denervated nictitating membrane has been shown to be hypersensitive in comparison with the innervated membrane to many agents other than adrenaline, and Burn and Trendelenburg (1954) re-investigated this question by perfusing the head of the cat. They found that the denervated membrane was regularly more sensitive than the normal membrane to adrenaline, noradrenaline and acetylcholine, but that both membranes were insensitive to other agents. They confirmed the evidence of Bacq and Fredericq (1935) that there are some cholinergic fibres in the sympathetic supply of the nictitating membrane. Thus the question arose whether the increased sensitivity of the denervated nictitating membrane to acetylcholine was due to a fall in cholinesterase. Burn and Philpot (1953) investigated this and found that both true and pseudocholinesterase were present in the nictitating membrane; they also found that in the denervated membrane there was a significant fall in true cholinesterase, but no similar fall in pseudocholinesterase. The observations thus combined to support the view that the hypersensitivity of the denervated nictitating membrane could be explained by a fall in the amount of the enzymes.

In the course of their work Burn and Philpot examined the cholinesterase in the iris after removal of the superior cervical ganglion. They expected that no change would be observed, but found that there was a fall in pseudocholinesterase. They pointed out the possibility that the hypersensitivity of the denervated iris to adrenaline might be related to this change rather than to the fall in amine oxidase described by Burn and Robinson (1952). A paper then appeared by Armin, Grant, Thompson and Tickner (1953) concerning the effect of denervation of the vessels of the rabbit ear. These authors found that degeneration of sympathetic fibres did not diminish the amine oxidase present in the vessels, but that it led to a reduction of the pseudocholinesterase to half the normal amount. They also showed that acetylcholine was present in the normally innervated vessel walls, and that this also disappeared after denervation. They suggested that the hypersensitivity to adrenaline was due to this disappearance.

While Burn and Robinson (1952) had investigated the change in amine oxidase in the denervated nictitating membrane very thoroughly, their evidence that a fall occurred in the denervated iris was much less strong, depending on only 7 experiments, the mean result being that the denervated membrane contained 77% of the activity of the normal membrane. The fall was conspicuous in only one experiment, and when this was omitted from the calculation the mean figure rose to 86%. Similarly, their conclusion that there was a fall in the amine oxidase in the denervated arteries was based (as they pointed out) on only 3 experiments. It was therefore decided to re-investigate the effect

of denervation on the amine oxidase in the iris and the arteries.

Methods

For observations on the iris the amine oxidase of the iris of the right eye was compared with that of the left eye in 8 normal cats, and also in 8 cats in which the superior cervical ganglion of one side had been removed in an aseptic operation 8 or 9 days previously. Both eyes were removed from the orbit and the cornea was cut off. The iris was then lifted out with a pair of fine forceps, and, after lightly draining on a piece of cork, was weighed on a watch glass. In earlier experiments 2 cats were used for each estimation. The two irides from the same side were chopped finely with scissors and suspended in phosphate buffer M/15 *p*H 7.4 to give a volume of 1.1 ml. 0.5 ml. was used in each manometer vessel (vol. 6 ml.); in addition each vessel contained KCN 0.1M, 0.07 ml., H_2O 0.06 ml., KOH/KCN, 0.1 ml. (centre compartment), and in the side arm either 0.07 ml. H_2O or tyramine 0.1M, 0.07 ml. Substrate was added after 10–15 min. temperature equilibration; the gas was O_2, temperature 37.5° C. Readings were taken over 30 min. In later experiments the right and left irides from a single cat were compared; the control without tyramine, which was found to be negligible, was omitted. In these experiments each iris was minced with a McIlwain chopper and transferred completely to the manometer vessel.

Cat arteries were treated similarly. The piece of artery from each side was chopped and the whole transferred to the manometer vessel, phosphate buffer M/15 *p*H 7.4, 0.45 ml., was added and the other reagents as described above. Readings were taken over 90 min. No control measurement of tissue in absence of substrate was found to be necessary.

For estimation of pseudocholinesterase in arteries the tissue was treated exactly as has been described for the amine oxidase estimations. The whole of the piece of artery of one forelimb was compared with the whole of the piece of artery from the opposite limb. The artery was chopped finely with a McIlwain chopper and then transferred to the manometer flask. The flask contained 0.7 ml. Krebs bicarbonate Ringer solution. The side arm contained 0.08 ml. 0.2M-butyrylcholine chloride solution. A control flask, containing the Ringer and substrate only, measured the spontaneous hydrolysis of butyrylcholine chloride. Gas mixture was nitrogen containing 5% CO_2, temperature 37.5° C. Readings were taken over 30 min.

Observations were made in which the volume changes in the two forelegs of a cat were compared after intravenous injections of adrenaline and of noradrenaline. The cats were those in which the stellate ganglion of one side had been removed 8 or 9 days previously in a sterile operation, using Anderson's method (1904). The cats were first anaesthetized with ether and then a spinal preparation made. The volume changes were measured by putting the two forelegs in plethysmographs of the pattern described by Dale and Richards (1918), and the volume changes were recorded by piston recorders. These were sufficiently sensitive to show the pulse of the limb with each heart beat.

Results

Amine Oxidase in Iris of Cat.—Results for the amount of amine oxidase in the normal and denervated iris are given in Table I. The figures for the right irides were expressed as a percentage of the corresponding figures for the left irides, and the mean percentage for normal right in terms of normal left was 98.4. When the figures for denervated right irides were expressed as a percentage of the corresponding figures for normal left irides, the mean percentage was 103.5. Thus the results indicated that removal of the superior cervical ganglion did not diminish the amount of amine oxidase in the iris.

TABLE I

AMINE OXIDASE IN IRIS EXPRESSED AS $\mu l.O_2/g./hr.$

Expt.	Normal Left	Normal Right	Denervated Right	Days Denervated	Right as % of Left
1	195	228	—	—	117
2	376	384	—	—	102
3	266	286	—	—	107
4	367	375	—	—	102
5	275	205	—	—	75
6	263	269	—	—	102
7	192	211	—	—	110
8	298	216	—	—	72
					Mean 98·4
9	224	—	270	8	120
10	331	—	427	8	129
11	410	—	375	9	91
12	325	—	246	9	76
13	164	—	252	9	154
14	294	—	274	9	93
15	370	—	321	9	87
16	426	—	342	9	80
					Mean 103·5

Amine Oxidase in the Foreleg Arteries.—Results for the amount of amine oxidase in the normal and denervated foreleg arteries are given in Table II. In the experiments in which the normally innervated vessels of the two forelegs were compared with one another the mean percentage for the right foreleg in terms of those of the left was 99.3. Similarly, the mean percentage for denervated vessels in terms of those of the corresponding normal vessels was 101.4 Thus the results indicated that removal of the stellate ganglion did not diminish the amount of amine oxidase in the foreleg arteries.

Pseudocholinesterase in the Foreleg Arteries.—As already described, Burn and Philpot found that extirpation of the superior cervical ganglion caused a fall in pseudocholinesterase in the iris, and Armin, Grant, Thompson and Tickner (1953) found that after denervation of the rabbit ear the

TABLE II
AMINE OXIDASE IN FORELEG ARTERIES OF CAT
$\mu l.O_2/g./hr.$

Expt.	Normal Left	Normal Right	Denervated Leg	Days Denervated	Result as % of Left or of Normal
17	162	170	—	—	105
18	176	181	—	—	103
19	105	94	—	—	90
					Mean 99·3
20	82	—	114	9	139
21	151	—	151	9	100
22	183	—	152	9	83
23	—	207	204	8	98
24				8	
25	—	145	126	8	87
26		166	189	9	114
					Mean 103·5

pseudocholinesterase present in the vessel walls declined in amount and often disappeared. We therefore made estimations of pseudocholinesterase in the arteries of the cat's foreleg. A comparison was made of the amounts present in normal right and normal left legs, the results of which are shown in Table III, Expts. 27–32. The mean result in six experiments was that the amount in the right leg was the same as the amount in the left leg. A comparison was then made after denervating one leg by extirpation of the stellate ganglion. Eight experiments were carried out in which denervation

TABLE III
PSEUDOCHOLINESTERASE IN ARTERIES $\mu l.CO_2/g./hr.$

Expt.	Normal Left	Normal Right	Denervated Leg	Days Denervated	Result as % of Left or Normal
27	854	1,280	—	—	150
28	1,625	1,170	—	—	72
29	1,430	1,580	—	—	111
30	1,375	870	—	—	63
31	2,255	2,015	—	—	89
32	780	1,090	—	—	140
					Mean 104
33	—	1,975	2,420	8	123
34	1,110	—	1,215	8	107
35	—	1,445	1,395	8	96
36	—	1,770	1,067	11	60
37	577	—	1,670	11	290
38	1,040	—	1,370	14	132
39	—	1,750	1,940	29	110
40	1,000	—	1,295	33	130
					Mean 131

was performed 8–39 days previously. The results showed no loss of pseudocholinesterase on the denervated side, and the mean figure for the denervated legs was 131% of that for the normal legs. Since Armin, Grant, Thompson and Tickner found that the fall in pseudocholinesterase was greater when the time interval after denervation was longer we decided to carry out further experiments at a longer interval.

Denervation and Constrictor Effect of Adrenaline.—The denervated vessels of the rabbit ear were found by Armin, Grant and their colleagues to be much more sensitive to the constrictor action of adrenaline than the normal vessels. In 1932 Burn described the effect of removing the stellate ganglion on the constriction produced in the vessels of the cat's foreleg in response to injections of ephedrine and tyramine. He observed that the constrictor action of these substances was lost, but also that " the constrictor action of adrenaline was less in the denervated than in the normal limb." Figs. 5, 6 and 7 in that paper each show this difference, though it was not large.

We therefore compared the response to adrenaline of the normal and denervated forelegs by enclosing them in plethysmographs, the cat having been prepared as a spinal animal. An example is shown in Fig. 1. The upper record is of the volume of the normal leg, while the middle record is that of the denervated leg. When 10 μg. adrenaline was injected, the constriction in the normal leg seen as the downward movement of the record

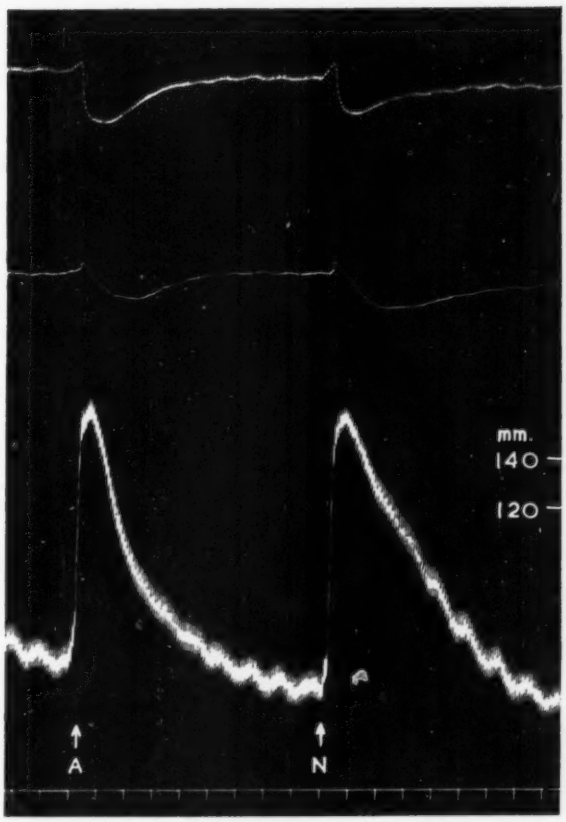

FIG. 1.—Upper record is the volume of the normal foreleg; middle record is the volume of the denervated foreleg; and lowest record is the blood pressure, in a spinal cat. The first injection is 10 μg. adrenaline, which caused more constriction in the normal foreleg than in the denervated. The second injection is 10 μg. noradrenaline, which caused about the same constriction in both forelegs.

was greater than that in the denervated leg. When 10 μg. noradrenaline was injected the constriction in the normal leg was about the same as that in the denervated leg.

In 10 cats in which the stellate ganglion of one side was removed from 35 to 55 days previously a series of injections of adrenaline and of noradrenaline was made, and the constrictions caused in the two legs were measured on the record afterwards. Mean values for these constrictions were then calculated, and the ratios of constriction in the normal leg to constriction in the denervated leg are shown in Table IV for both adrenaline and noradrenaline. At the end of each experiment the arteries were dissected and the pseudocholinesterase present was estimated. These results are also shown in Table IV together with the ratio of the amount present in the denervated leg to that in the normal leg. Thus the pseudocholinesterase ratios are expressed as $\frac{\text{denervated}}{\text{normal}}$, while the adrenaline constriction ratios are expressed as $\frac{\text{normal}}{\text{denervated}}$, for if the amount of pseudocholinesterase in some way restricts the adrenaline constriction then the ratios so expressed should show a correspondence.

TABLE IV

PSEUDOCHOLINESTERASE RATIOS AND CONSTRICTION RATIOS

Expt.	Pseudo ChE		ChE Ratio D/N	Constriction Ratio N/D	
	Normal	Denervated		Adrenaline	Noradrenaline
41	660	685	1·04	0·87	0·42
42	941	745	0·79	1·32	0·4
43	462	1,170	2·53	3·5	1·03
44	1,042	1,090	1·05	1·3	0·94
45	830	795	0·96	0·81	1·0
46	908	1,020	1·12	1·86	1·29
47	1,240	881	0·71	1·1	0·15
48	521	623	1·2	3·75	1·15
49	1,400	1,310	0·94	0·59	0·53
50	761	781	1·03	1·65	0·53
		Mean	1·14	1·67	0·74

Table IV shows figures for pseudocholinesterase similar to those in Table III and also that adrenaline caused more constriction in the normal leg than in the denervated in 7 of 10 experiments. The reverse was true of noradrenaline, which usually caused less constriction of the normal leg than of the denervated.

There appeared to be some degree of correlation between the amounts of pseudocholinesterase in the two legs and the responses to adrenaline. That is to say, the legs which constricted less in response to an injection of adrenaline were those in which the arteries were found to have more pseudocholinesterase.

When the figures in the fourth and fifth columns of Table IV were examined by the formula given by Fisher (1946), it was found that $r=0.6989$, $t=2.75$ and P was 0.025. Thus there was a correlation between the fourth and fifth columns of Table IV which was significant at the 2.5% level.

Observations with Enzyme Inhibitors.—Some observations have been made on the action of substances known to inhibit amine oxidase and cholinesterase. Marsilid (isopropylisoniazide) has been shown by Zeller and Barsky (1952) to inhibit amine oxidase and not to inhibit cholinesterase. Its effect was tested on the blood pressure and nictitating membrane of the spinal cat, and also in the perfused head of the cat (Burn and Trendelenburg, 1954). Injections of adrenaline and noradrenaline were made before and after injecting marsilid in amounts varying from 5 to 500 μg. The action of adrenaline on the blood pressure and on the nictitating membrane was not modified by these injections either in the spinal cat (4 expts.) or in the perfused head (5 expts.). In 2 of 5 experiments on the blood pressure the action of noradrenaline was increased after injecting marsilid, but these increases were much less than those regularly produced by the injection of cocaine.

The action of anticholinesterases was different. They were observed to diminish the pressor action of adrenaline and to increase its action on the nictitating membrane. An example is shown in Fig. 2 in which the injection of 0.8 mg. eserine sulphate had these effects. After the injection of

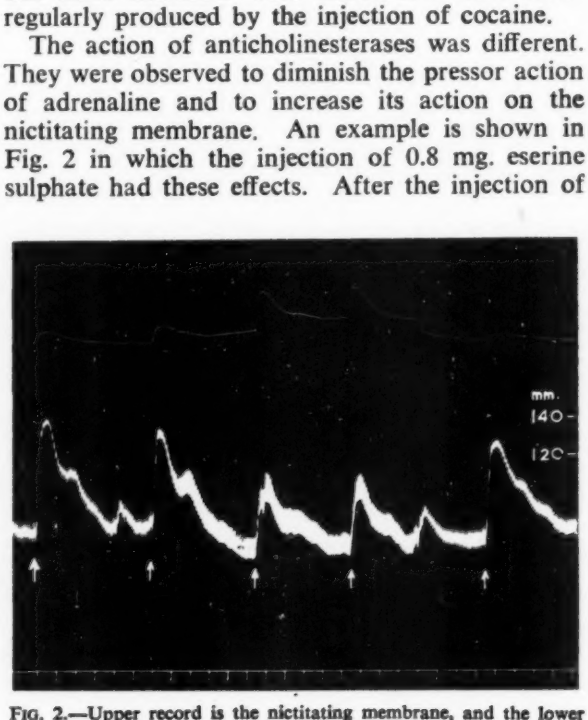

FIG. 2.—Upper record is the nictitating membrane, and the lower record is the blood pressure in a spinal cat. At each arrow 8 μg. adrenaline was injected. Between the first and second arrows 0.8 mg. eserine sulphate was injected. and between the fourth and fifth arrows 1.4 mg. atropine sulphate was injected.

1.4 mg. atropine sulphate the action of adrenaline on the nictitating membrane was almost abolished, while the pressor action was restored and sometimes increased. Neostigmine and Nu 683 (the dimethyl carbamate of (2 - hydroxy - 5 - phenyl - benzyl) - trimethylammonium bromide) were found to have a similar action to eserine. The amounts of these anticholinesterases which augmented the effect of adrenaline on the nictitating membrane were enough to cause fasciculation in skeletal muscle, but the augmentation was still evident after the injection of decamethonium up to 0.5 mg. to stop the fasciculation. When atropine was given first, in a dose of 2 mg., the augmentation was not seen.

Discussion

The observations have failed to confirm the previous work of Burn and Robinson (1952) that degeneration of the sympathetic supply leads to a fall in the amount of amine oxidase present in the iris and in the foreleg arteries of the cat. They came to the conclusion that such a fall took place on the basis of too few experiments because they had observed a fall in many experiments on the nictitating membrane.

The fact that amine oxidase in the iris is not affected by degeneration of the sympathetic fibres underlines the possibility mentioned by Burn and Philpot (1953) that the increased sensitivity to adrenaline caused by this degeneration depends on the fall in pseudocholinesterase which they recorded. This point needs further investigation in the isolated iris, in which the effect of anticholinesterases on the response to adrenaline could be studied.

In the foreleg arteries degeneration of the sympathetic fibres has not been found to cause a diminution either in the amount of amine oxidase or of pseudocholinesterase. A measurement has been made of volume changes in the normal and denervated forelegs both when adrenaline and noradrenaline were injected. Only in one of ten cats was the denervated foreleg more sensitive to the action of adrenaline than the normal foreleg, and in four it was much less sensitive. When the relative constriction in the two forelegs of each cat was compared with the relative amounts of pseudocholinesterase, it was found that there was a significant correlation between them.

These results point in the direction of the conclusions of Grant and Thompson and their colleagues that the response of blood vessels to adrenaline may be determined by the activity of a system in their walls which produces acetylcholine. If the constriction produced by adrenaline is limited by the acetylcholine present, then when there is much acetylcholine the constriction will be small. If the assumption is made that the activity of this acetylcholine system is proportional to the amount of pseudocholinesterase, then we have a basis for the correlation found between the constriction ratio of normal and denervated limbs in response to adrenaline and the ratio of the amounts of pseudocholinesterase present.

The effects of cholinesterase inhibitors in diminishing the pressor action of adrenaline and that of atropine in restoring it are also consistent with this explanation. So also is the potentiation of the action of adrenaline on the nictitating membrane by anticholinesterases, for both adrenaline and acetylcholine cause contraction of the nictitating membrane ; on this structure they do not oppose one another as in the blood vessels.

Griffin, Green, Youmans and Johnson (1954) have studied the changes in resistance which occur in the vessels of the dog's leg when adrenaline and noradrenaline are injected, comparing the results in normally innervated and previously denervated legs. They made observations on the muscle vessels and skin vessels separately. In the muscle vessels the effect of adrenaline was practically the same in the normal and previously denervated legs. The effect of noradrenaline was greater in the denervated legs, but the range of variation in the denervated legs was such that the difference was not statistically significant. In the skin vessels the effect of adrenaline was greater in the denervated legs by a significant amount. The effect of noradrenaline was also greater there, but again not significantly greater because of the wide spread. These findings appear to agree with the results of Armin, Grant, Thompson and Tickner in the rabbit ear vessels since these vessels are skin vessels, and they also agree with the results in the present paper since the volume changes recorded by the plethysmograph probably represent the reaction of muscle vessels. If this suggestion is correct it follows that denervated muscle vessels may not become more sensitive to adrenaline as do denervated skin vessels.

Burn and Robinson (1952) considered that the effect of cocaine in increasing the tone of the normal nictitating membrane, though not affecting that of the denervated membrane, was due to inhibition of amine oxidase. Since we have found that marsilid has no similar effect, it is doubtful whether their conclusion was correct. In view of Schayer and Smiley's evidence (1953) that the labelled carbon of the $-N.CH_3$ group was quanti-

tatively excreted in the urine of rats to which marsilid had previously been administered, it seems clear that a considerable proportion of adrenaline is destroyed in the body of the rat by amine oxidase, and we were therefore surprised to observe that marsilid did not potentiate effects of adrenaline. Smith and Alperi (1954) have studied the effect of amine oxidase inhibitors on isolated carotid arteries from swine and from dogs. They came to the conclusion that, while amine oxidase appears to play an important part in the arteries of swine, it has no importance in the arteries of the dog.

The effect of degeneration of sympathetic fibres on the response of the foreleg arteries to noradrenaline differed from that to adrenaline, since the denervated leg was more sensitive to noradrenaline in five of ten cats, and only in one was it rather less sensitive. This difference in the response of the two legs to noradrenaline cannot be related to such enzyme changes as we have observed, and must have some other explanation. We have none to put forward, but something about the difference between noradrenaline and adrenaline may be said.

Bülbring and Burn (1938) found that the response of the normal and denervated nictitating membranes varied for different sympathomimetic amines. The proportion of cats in which the denervated membrane responded less than the normal membrane to higher doses of these amines increased the more the structure of the amine differed from that of the transmitter. One of us (J. H. B.) suggested at the pharmacological meeting after the International Congress in Copenhagen (1950) that, while we think of the relation of stimulant substance and receptor as that of key and lock, it may be that normally the lock is capable of adjusting itself to many keys, but that after degeneration of the nerve supply it tends to lose this power of adjustment, and continues to be fitted perfectly only by the transmitter itself. Thus might be explained the diminished sensitiveness of the denervated foreleg arteries of many cats to adrenaline, while the sensitivity to noradrenaline remains greater than normal. This would agree with the observation (Burn, 1932) that tyramine failed to cause any constriction in the denervated limb. The hypersensitivity to noradrenaline, however, is not explained in these terms.

Summary

1. Further observations have been made on the effect of degeneration of sympathetic fibres on the amount of amine oxidase in the iris of the cat's eye and in the foreleg arteries of the cat. Degeneration of the sympathetic fibres does not cause a change in the amount of amine oxidase in either organ.

2. Degeneration of the sympathetic fibres to the cat's foreleg arteries does not reduce the amount of pseudocholinesterase present, nor does it make the foreleg more sensitive to the constrictor action of adrenaline.

3. In most experiments the constrictor response of the denervated foreleg to adrenaline was less than that of the normal foreleg, and the ratio of the two constrictor responses was found to be inversely related to the ratio of the amounts of pseudocholinesterase present.

4. The amine oxidase inhibitor, marsilid, did not modify the response of the nictitating membrane to adrenaline, nor the pressor response to adrenaline in the spinal cat. Cholinesterase inhibitors, however, diminished the pressor response to adrenaline and augmented the contraction of the nictitating membrane caused by adrenaline.

5. Degeneration of the sympathetic fibres to the cat's foreleg arteries usually makes the foreleg more sensitive to the constrictor action of noradrenaline.

This work has been done by F. J. P. during the tenure of an I.C.I. Fellowship, and by U. T. during the tenure of a British Council Scholarship.

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HYDROLYSIS OF SUCCINYLCHOLINE SALTS

BY

P. J. FRASER

WITH AN ADDENDUM BY TUDOR S. G. JONES AND R. H. PAIN

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Glick (1941), whilst working with various esters of choline, demonstrated that the choline salt of succinic acid was hydrolysed by horse serum and by alkali. Bovet, Bovet-Nitti, Guarino, Longo, and Marotta (1949) and de Beer, Castillo, Phillips, Fanelli, Wnuck, and Norton (1951) showed that succinyldicholine possessed powerful neuromuscular-blocking properties. The latter authors showed that eserine potentiated the paralysis of the cat gastrocnemius caused by succinyldicholine. As compound 49-164, which does not inhibit cholinesterase, produced a similar potentiation they doubted whether cholinesterase was the enzyme responsible for the destruction of succinyldicholine. This seemed worth re-investigation.

In the last two or three years many surgeons and anaesthetists have preferred succinyldicholine to other neuromuscular-blocking compounds for many surgical procedures because of its very short duration of action and its freedom from undesirable side-effects. It is ideally suited for administration as an intravenous drip to give a rapidly controllable degree of muscle relaxation. It has also found favour for use with thiopentone in electroconvulsion therapy (Arnold, Böck-Greisau, and Ginzel, 1951 ; Richards and Youngman, 1952).

Mayrhofer (1952) has, however, stated that succinyldicholine is stable in solution for several weeks outside the refrigerator and also when mixed with thiopentone. This was surprising, since succinyldicholine is unstable in alkaline solution (Glick, 1941) and the *p*H of thiopentone solution is around 11.0. It thus seemed desirable to re-examine Mayrhofer's claims. The hydrolysis of succinyldicholine at elevated temperatures, in alkaline solutions, and by pseudo-cholinesterase, has accordingly been studied. A quantitative comparison has also been made between succinyldicholine and its first hydrolysis product, succinylmonocholine, which still preserves a degree of neuromuscular-paralysing activity.

METHODS

Toxicity.—Groups of 5 or 10 albino mice of either sex were used, injections being made into the tail vein.

Action on Nerve-Muscle Preparations.—The isolated rat diaphragm–phrenic nerve preparation of Bülbring (1946) was used (*a*) as an *in vitro* test for concentration of succinyldicholine, and (*b*) for comparison of the concentrations of succinyldicholine and succinylmonocholine to give equal neuromuscular-paralysing action. The isolated organ was immersed in a Ringer-Locke solution in a 100 ml. bath, and square stimuli of 2 V. and of 0.23 msec. duration were applied at 5/min. to the nerve. Drugs were added from a tuberculin syringe and left in contact with the diaphragm for 3 min. This was followed by two washes with Ringer-Locke at 3 and 6 min. respectively, giving a total cycle of 10 min. In 3 min., 50% paralysis of the diaphragm twitch was produced by a concentration of about 15.0 μg./ml. of succinyldicholine.

Tests *in vivo* were performed on cats and rabbits of either sex. The cats were anaesthetized with pentobarbitone sodium (usually 40 mg./kg. i.p.), and the rabbits with urethane (amount required varied considerably). Respiration was recorded from the cannulated trachea by a tambour recorder. The blood-pressure tracing was obtained from the cannulated carotid artery and injections were made into the femoral vein (cat) or jugular vein (rabbit). The muscle—gastrocnemius in the cat, extensor digitorum longus in the rabbit—was stimulated via the sciatic nerve by 6 discharges/min. from a neon stimulator.

For the chick paralysis test of Buttle and Zaimis (1949) two- to three-week-old chicks of either sex were used, injections being made into the wing vein. The duration of paralysis was recorded.

Manometric Methods (Warburg Respirometer).—Single side-arm flasks were used. The substrate was placed in the side arm and the enzyme preparation and Ringer-bicarbonate, to a total volume of 3.0 ml., in the base of the flask. Reactions were allowed to proceed in an atmosphere of 95% N_2 : 5% CO_2. Corrections were made for extraneous temperature and pressure variations and for spontaneous hydrolysis of the substrate. The results were recorded as μl. CO_2 evolved at 37° C. Ringer-bicarbonate was prepared by mixing

together, immediately before use, 50.0 ml. 0.9% NaCl, 15.0 ml. 1.26% $NaHCO_3$, 1.0 ml. 1.2% KCl and 1.0 ml. 1.76% $CaCl_2$ (hydrated), and gassing for 10 min. with a 95% N_2: 5% CO_2 mixture.

Cholinesterase Preparations. — Human blood was withdrawn into a dry syringe and 1 part of a saturated solution of potassium oxalate was immediately added to 10 parts of whole blood. After gently mixing, this was centrifuged and the supernatant plasma drawn off. This provided the human plasma (pseudo) cholinesterase preparation which produced about 1,500 μl./CO_2/ml./hr. with benzoylcholine chloride (0.015M) as substrate. The packed red cells were washed twice with Ringer-bicarbonate, centrifuged, and the supernatant fluids discarded. The packed, washed red cells were then haemolysed by adding distilled water up to the original blood volume. This provided the human red blood-cell (true) cholinesterase preparation which produced about 2,000 μl. CO_2/ml./hr. with acetyl-β-methylcholine chloride (0.03M) as substrate.

Succinylcholine Salts.—Succinyldicholine dichloride was prepared by Burroughs Wellcome and Co. Succinylmonocholine monochloride was kindly prepared for me by Dr. Tudor Jones of the Wellcome Chemical Research Laboratories (see addendum).

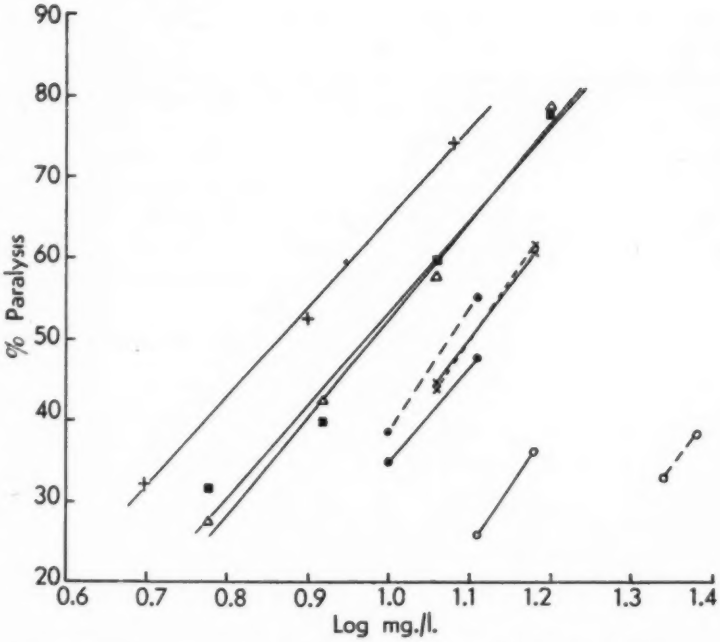
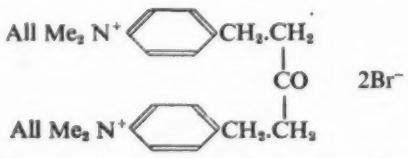


FIG. 1.—Dose-response curve for succinyldicholine on isolated rat diaphragm. Ordinate: % paralysis of diaphragm in 3 min. Abscissa: log mg./l. succinyldicholine. Three dose-response curves and three pairs of 2×2 assays of "standard" against "test" are given. These were chosen at random from over 50 examples and have a mean slope of 132, i.e., 132% change in paralysis per tenfold dose increment.

284*C*51.—This compound (Copp, 1953) is 1: 5-bis(4-allyldimethylammoniumphenyl)-*n*-pentan-3-one dibromide,



It has been shown by Fulton and Mogey (1954) to be a specific inhibitor of true cholinesterase with a *p*I 50 of 7.8 for true cholinesterase and a *p*I 50 of less than 3.0 for pseudo-cholinesterase.

RESULTS

Dose–Response Curve (Rat Diaphragm).—The log dose–response curve for succinyldicholine on the rat diaphragm was substantially linear over a wide range of responses. Fig. 1 gives 6 such curves chosen at random from over 50 examples. Three of these were multipoint log dose–response curves and three were pairs of curves taken from 2 × 2 assays of "test" against "standard." The mean slope of these curves was 132 (i.e., 132% change in paralysis/tenfold dose increment). Calculation from this mean slope showed that a 50% fall in concentration of succinyldicholine was accompanied by a 40% lowering of paralysis.

Effect of p*H*.—Succinyldicholine solutions were buffered at *p*H 5.0, 7.4, 8.2, 8.95, and 9.5 and maintained at 21° C. The residual succinyldicholine activities of the various solutions were compared with that of the *p*H 7.4 solution on the isolated rat diaphragm–phrenic nerve preparation. The amount of control succinyldicholine chosen was such as to give 70% paralysis in 3 min.

The difference between the % paralysis caused by the test and by the control solutions was plotted against the time in min. after solution of the succinyldicholine in buffer (Fig. 2). Equivalent amounts of the buffers alone when added to the organ bath produced no change in the size of the muscle twitch.

These results showed no detectable hydrolysis of succinyldicholine at *p*H 5.0, or at *p*H 7.4, but a slow hydrolysis at *p*H 8.2. In more alkaline solutions hydrolysis was more rapid, and at *p*H 9.5 there was a 50% loss of activity in just under 3 hr.

Hydrolysis in Thiopentone Solution.—Thiopentone alone on the isolated rat diaphragm caused a very marked increase in the size of the muscle twitch and in the sensitivity of the preparation to paralysis by succinyldicholine. In view of this it was necessary to remove the thiopentone from a mixture of the two drugs before a satisfactory test of succinyldicholine hydrolysis by thiopentone could be performed on the rat diaphragm.

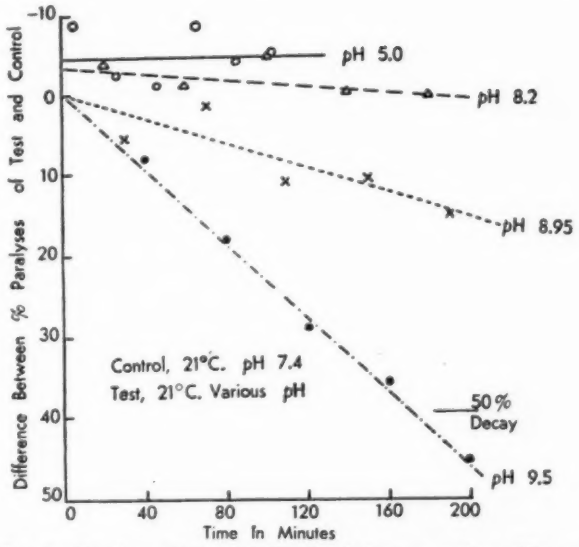


FIG. 2.—To show effect of buffered solutions at various *p*H's on destruction of succinyldicholine *in vitro*. Rat diaphragm (isolated). Ordinate: difference between % paralysis caused by control and test samples of same initial concentrations. Abscissa: time in min. after preparation of solutions.

A solution in distilled water was prepared, each ml. containing 3.0 mg. succinyldicholine and 10.0 mg. thiopentone. The *p*H of this mixture was 11.0 and it was maintained at room temperature (20° C.) until aliquots were neutralized to *p*H 7.0 with HCl. This resulted in precipitation of the thiopentone which was filtered off. (A control run in the absence of succinyldicholine showed that sufficient thiopentone had been removed for such as remained to have no effect on the diaphragm.)

Half a ml. of filtrate was then added to the 100-ml. organ-bath and the paralysis occurring in 5 min. was noted. This concentration was found to give total paralysis of the diaphragm in 5 min. when neutralization was performed immediately after preparation of the solution. No paralysis was obtained when 90% of this amount of succinyldicholine had been hydrolysed. The plot of % paralysis against log time of precipitation of the thiopentone after mixing of the two drugs, gave the straight line shown in Fig. 3. When a similar procedure was adopted using NaOH to *p*H 11.0 instead of thiopentone, total hydrolysis was obtained in under 10 sec. The significance of this will be discussed later.

Effect of Temperature.—In a series of tests similar to those for demonstrating the effect of *p*H, test and control solutions were buffered at *p*H 7.4. The control solution was maintained at 21° C. and the test solutions were incubated at temperatures of 40° C., 50° C., and 100° C. respectively.

Fig. 4 presents the mean results and demonstrates the instability of succinyldicholine at temperatures higher than normal body temperature, 50% hydrolysis occurring in about 30 min. at 100° C.

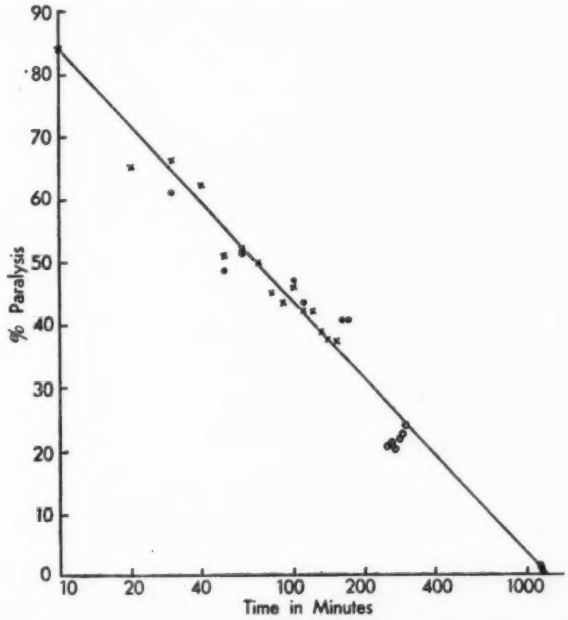


FIG. 3.—To show decay of succinyldicholine in the presence of thiopentone (10.0 mg./ml.) *p*H 11.0. X, O, ●—three rat diaphragms. Ordinate: % paralysis. Abscissa: log time in min. between mixing of thiopentone and succinyldicholine and precipitation of thiopentone by HCl to *p*H 7.0.

Effect of Cholinesterases.—The action of cholinesterases on the paralysing activity of succinyldicholine was also studied. For the control, succinyldicholine was buffered at *p*H 7.4 and maintained at 37° C. Test samples were made up in plasma, red blood cell haemolysate, whole blood, or in plasma to which eserine sulphate had been added to a final concentration of 10^{-5}M and also incubated at 37° C.

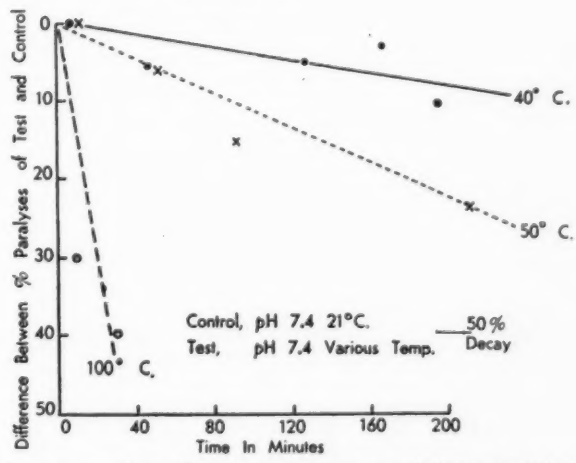


FIG. 4.—To show effect of temperature on solutions of succinyldicholine buffered at *p*H 7.4. Rat diaphragm (isolated). Ordinate and abscissa as in Fig. 2.

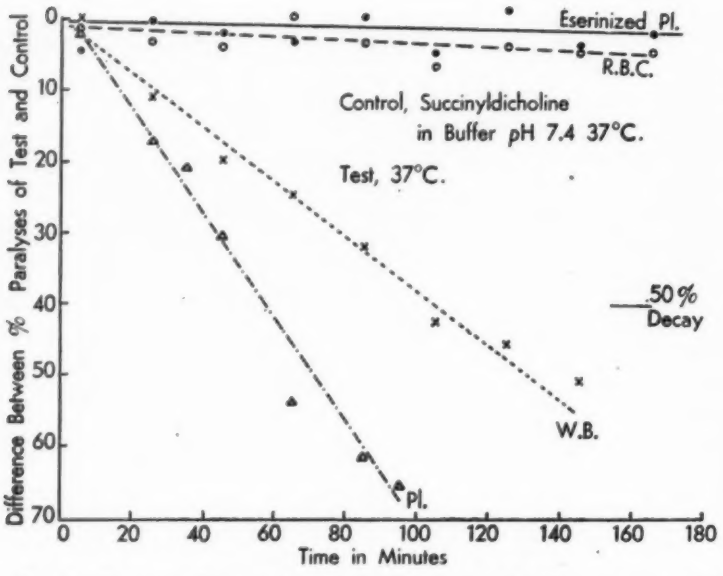
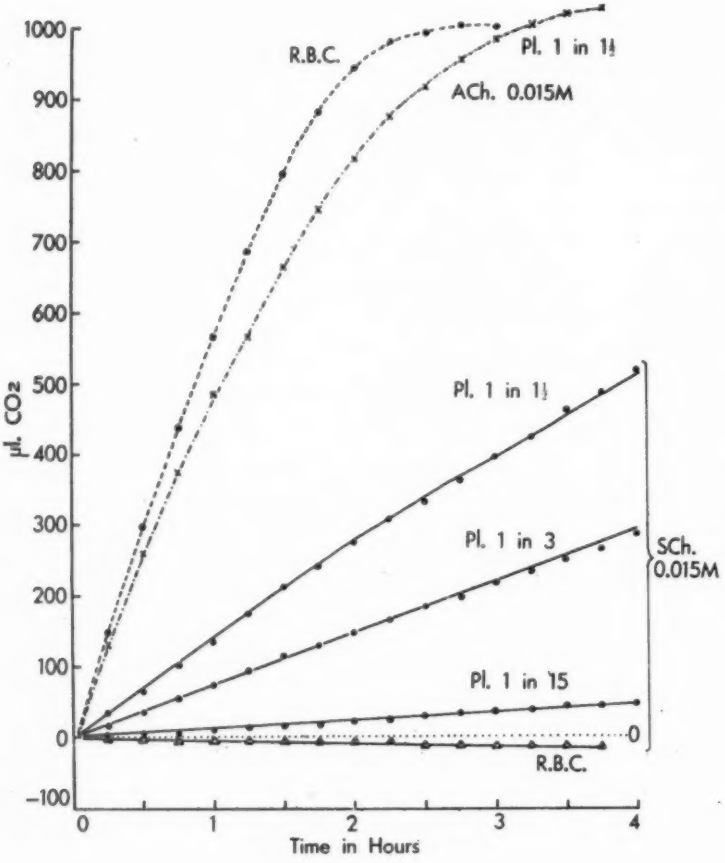


FIG. 5.—To show effect of cholinesterases on succinyldicholine *in vitro*. Rat diaphragm (isolated). Ordinate and abscissa as in Fig. 2. Pl, human plasma (pseudo-cholinesterase). W.B., human whole blood (true and pseudo-cholinesterases). R.B.C., human red blood cell haemolysate (true cholinesterase). Eserinized Pl, human plasma pseudo-cholinesterase inhibited by eserine sulphate (10^{-5}M).



Neither red blood cells nor eserinized plasma destroyed succinyldicholine (Fig. 5). Uninhibited plasma caused rapid hydrolysis of succinyldicholine, whereas whole blood acted at a somewhat slower rate. The plasma volume in whole blood is 50–60% and, from Fig. 5, the rate of hydrolysis in whole blood was 60% of that in plasma. These results show that, *in vitro*, succinyldicholine was hydrolysed by pseudo-cholinesterase but was unaffected by either true or inhibited pseudo-cholinesterase.

This was also demonstrated in the Warburg respirometer using three concentrations of human plasma, human red blood cell haemolysate, and eserinized plasma. In Fig. 6 the hydrolysis curves for acetylcholine chloride have been included for comparison.

Cat Gastrocnemius.—Rapid destruction of succinyldicholine *in vivo* was shown by the short duration of paralysis of the cat gastrocnemius muscle on intravenous injection of the drug. Further, identical injections repeated at 15-min. intervals caused the same degree (%) and duration of paralysis after the first few injections; hence there was no evidence of accumulation of succinyldicholine in the animal.

In the experiment shown in Fig. 7, the cat was given a series of injections of succinyldicholine (0.04 mg./kg.) until a constant response was obtained. At the arrow B, 0.2 mg./kg. 284C51 was given intravenously. This was sufficient to cause a considerable depression of true cholinesterase activity in the blood stream but to have no effect on the pseudo-cholinesterase. Following this by the standard injection of succinyldicholine there was no change from the response obtained before 284C51. After 0.2 mg./kg.

FIG. 6.—To show hydrolysis of succinyldicholine by cholinesterases *in vitro*. Warburg respirometer. Upper two curves show hydrolysis of acetylcholine (0.015M) by human plasma and red blood cells. Lower four curves show hydrolysis of succinyldicholine (0.015M) by 3 concentrations of human plasma, and lack of hydrolysis by human red blood cells. Ordinate: corrected output of CO_2 in µl. Abscissa: time in hr.

eserine sulphate, which gave 40% inhibition of the pseudo-cholinesterase, the paralysis caused by the standard dose of succinyldicholine was increased and prolonged. Normal cat plasma had half the pseudo-cholinesterase activity of human plasma. Fig. 8 presents the results of a typical experiment, and shows how the maximum effect of the eserine in potentiating the succinyldicholine was not achieved until about 1 hr. after the eserine was injected intravenously, despite the fact that this dose of eserine caused its maximum inhibition of the plasma pseudo-cholinesterase within 10 min. of the injection.

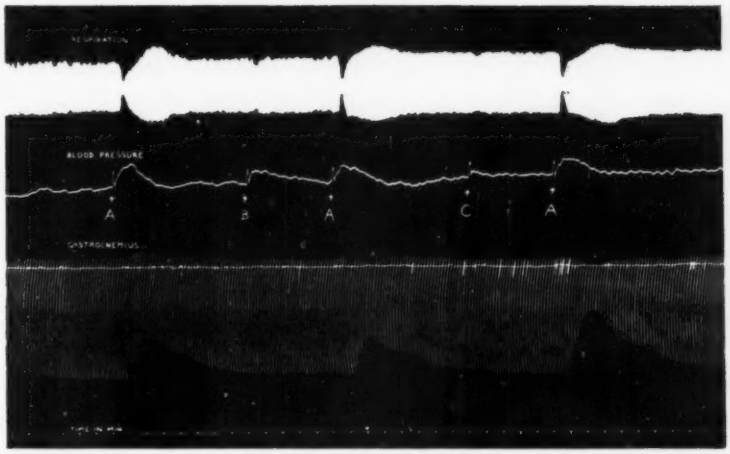


FIG. 7.—To show the potentiation of the succinyldicholine paralysis of the cat gastrocnemius *in vivo* by eserine sulphate. Cat ♀, 5 kg. pentobarbitone sodium (40 mg./kg. i.p.). Gastrocnemius stimulated through sciatic nerve. At A, succinyldicholine 0.04 mg./kg. i.v. At B, 284C51 0.2 mg./kg. i.v. At C, eserine sulphate 0.2 mg./kg. i.v. (see also Fig. 8).

Chick Paralysis.—Intravenous injection of succinyldicholine into the chick causes a spastic paralysis of the leg and neck muscles (Buttle and Zaimis, 1949). Using a dose of 0.03 mg./kg., a spastic paralysis lasting 3–4 min. was obtained. 0.5 mg./kg. 284C51 given half an hour before the succinyldicholine produced no change in the duration of paralysis from that found in the controls. Similarly, eserine sulphate at 0.1 mg./kg. and 0.2 mg./kg. caused no significant prolongation of paralysis; but when 0.5 mg./kg. was given half an hour before the succinyldicholine the duration of paralysis was extended to 7–8 min.—an increase of 80–100%. Normal chick plasma had 1/18 of the pseudo-cholinesterase activity of human plasma with benzoylcholine as substrate, and was also capable of hydrolysing succinyldicholine at a slow rate, as is shown in Fig. 9.

Recovery in Rabbits.—Bourne, Collier, and Somers (1952) and Evans, Gray, Lehmann, and Silk (1952) have demonstrated a low pseudo-cholinesterase level in the plasma of patients who

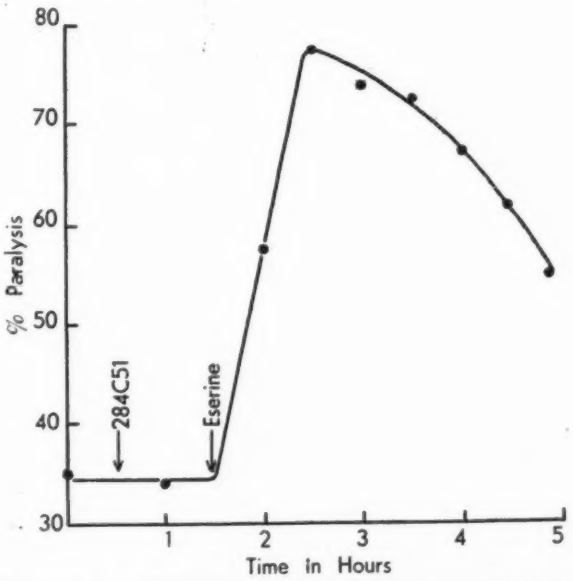


FIG. 8.—Graphical presentation of potentiation of succinyldicholine paralysis of cat gastrocnemius by eserine. Ordinate: % paralysis of gastrocnemius muscle. Abscissa: time in hr. At first arrow: 284C51 0.2 mg./kg. i.v.; at second arrow: eserine sulphate 0.2 mg./kg. i.v. Each dot represents % paralysis of the muscle caused by 0.04 mg./kg. succinyldicholine i.v.

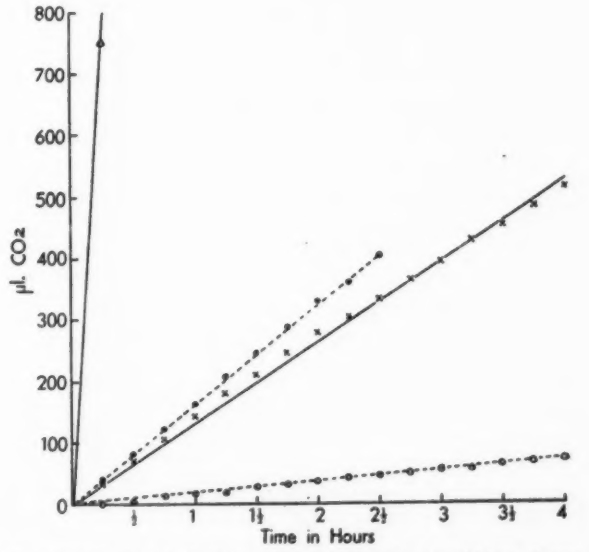


FIG. 9.—To show hydrolysis of succinyldicholine by chicken plasma. Warburg respirometer. Ordinate: µl. CO_2. Abscissa: time in hr. △——△ Human plasma (1 in 1¼)+benzoylcholine (0.015M). X——X Human plasma (1 in 1¼)+ succinyldicholine (0.015M). ●- - - -● Chicken plasma (1 in 1½)+benzoylcholine (0.015M). O- - - -O Chicken plasma (1 in 1½)+succinyldicholine (0.015M).

had unusually prolonged recovery times from succinyldicholine apnoea. In view of the findings of Koelle (1953), that rabbits fall into two distinct groups on the basis of their plasma pseudocholinesterase levels, rabbits were used to determine whether the low-activity group would take longer to recover from succinyldicholine paralysis than the high-activity group. Some 20 rabbits from each group were used and the extensor digitorum longus muscle was stimuated via the sciatic nerve. It was not possible to demonstrate any relationship between plasma pseudo-cholinesterase level, sensitivity to succinyldicholine, and time for recovery of the muscle from 66% paralysis to 33%. Enzyme estimations performed on this muscle failed to show any appreciable pseudo-cholinesterase activity.

Succinylmonocholine Monochloride.—This compound, which is the first hydrolysis product of succinyldicholine, had similar qualitative actions. Quantitatively, however, it differed, the ratio of activities varying according to the test animal (Table I).

TABLE I

COMPARISON OF ACTIVITIES OF SUCCINYLDICHOLINE AND SUCCINYLMONOCHOLINE

Test Animal	Criterion for Comparison	Dose of Drug		Ratio of Activity of Succinyldicholine to Succinylmonocholine
		Succinyldicholine	Succinylmonocholine	
Mouse Rat	i.v. LD50 (mg./kg.)	0·455	4·66	10: 1
Cat	50% paralysis diaphragm in 3 min. (mg./100 ml.) ..	0·9	10·0	11: 1
Chick	50% paralysis of gastrocnemius muscle (mg./kg.)	0·025	1·0	40: 1
	3–4 mins. duration spastic paralysis (mg./kg.) ..	0·03	15·0	500: 1

In the Warburg respirometer, succinylmonocholine was hydrolysed by human plasma pseudocholinesterase (see Fig. 10). The ratio of the rate of hydrolysis of succinyldicholine to that of succinylmonocholine was 1.4. No hydrolysis was obtained with human red blood cells or with eserinized plasma (eserine 10^{-5} M).

Discussion

Using the isolated rat diaphragm, it has been shown that succinyldicholine is unstable in alkaline solutions and at temperatures above about 40° C. Increasing alkalinity or increasing temperature led to a faster rate of destruction of the succinyldicholine. In Figs. 2 and 4, summarizing these results, a straight line was obtained by plotting % paralysis of the rat diaphragm against incubation time. Fig. 1 showed that, on the rat diaphragm, % paralysis was directly proportional to log concentration, and hence the hydrolysis of succinyldicholine both by alkali and by heat, was a first-order reaction. The same deduction also applied to the hydrolysis by pseudo-cholinesterase (Fig. 5). The reaction with thiopentone was different. Firstly, the rate of destruction of succinyldicholine by thiopentone at *p*H 11.0 was very much less rapid than that obtained with NaOH at *p*H 11.0 ; secondly (Fig. 3), a straight line was only obtained when % paralysis was plotted against log time—thus this was a second-order

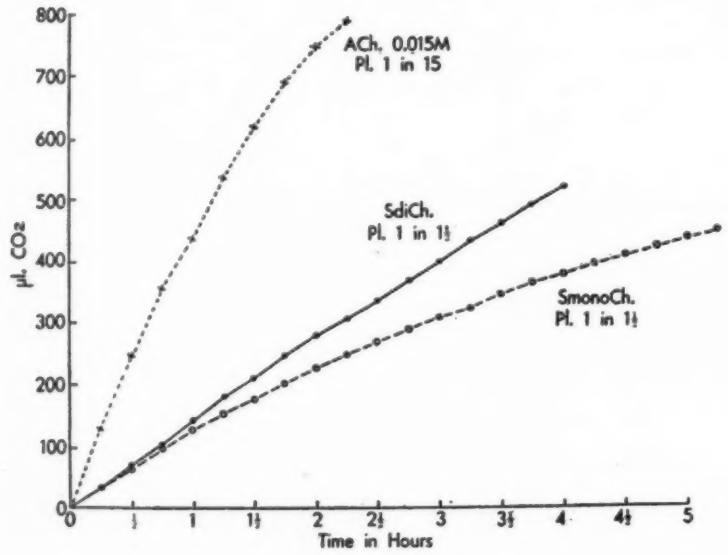


FIG. 10.—To show hydrolysis of succinylmonocholine by pseudo-cholinesterase *in vitro*. Warburg respirometer. Ordinate: µl. CO_2. Abscissa: time in hr. X----X Human plasma (1 in 15)+acetylcholine (0.015M). ●——● Human plasma (1 in 1½)+succinyldicholine (0.015M). O----O Human plasma (1 in 1½)+succinylmonocholine (0.015M).

reaction. The result was suggestive of a reaction occurring between the succinyldicholine and the thiopentone as well as the straightforward alkaline hydrolysis. The rate of 50% loss of activity in the first 90 min. was sufficiently fast to make it inadvisable to mix thiopentone and succinyldicholine in the one syringe for intravenous injection.

The *in vitro* enzyme experiments agreed with those of previous authors (Glick, 1941 ; de Beer *et al.*, 1951 ; Whittaker, 1951 ; Evans *et al.*, 1952) that succinyldicholine salts were destroyed by pseudo-cholinesterase, but differed from those of Bovet-Nitti (1949) and Tammelin (1953), who demonstrated hydrolysis by both cholinesterases. *In vivo* experiments in the cat and chick also showed that destruction of the true cholinesterase had no effect on the sensitivity to succinyldicholine, whereas inhibition of pseudo-cholinesterase considerably potentiated the paralysis caused by a standard injection of the drug. Further, in the cat, maximum inhibition of pseudo-cholinesterase in the plasma occurred in under 10 min. after intravenous injection of 0.2 mg./kg. eserine sulphate. However, maximum potentiation of succinyldicholine did not occur until 1 hr. after the injection of eserine (Fig. 8). It thus appeared that the pseudo-cholinesterase level in the plasma was not the only factor determining the duration of action of succinyldicholine *in vivo* (cf. de Beer *et al.*, 1951). Further support was provided by the rabbit experiments, where it was not possible to demonstrate any relationship between plasma pseudo-cholinesterase level, sensitivity to succinyldicholine and time for recovery of the extensor digitorum longus muscle from 66% paralysis to 33%. Enzyme estimations performed on this muscle showed only slight pseudo-cholinesterase activity. It is suggested that, although the plasma enzyme level plays an important part in determining the effect of an injection of succinyldicholine, other factors—for example, the pseudo-cholinesterase concentration in the muscle—may be equally important.

Succinylmonocholine was similar in its actions to succinyldicholine but much less active (Table I). The ratio of 40:1 on the cat gastrocnemius was confirmed by Collier and Macauley (1953) on the cat tibialis. In the hydrolysis by human plasma pseudo-cholinesterase (Fig. 10) there was a slight discrepancy between these results and those reported by Lehmann and Silk (1953). Calculation from Lehmann and Silk's figures gave a ratio of the rate of hydrolysis of succinyldicholine to that of succinylmonocholine, of 1.67 at 0.055M concentration of substrates, and of 3.8 at 0.0055M, as compared with 1.4 at substrate concentrations of 0.015M in the experiments reported here. The concentration of enzyme used was, however, approximately three times greater in Lehmann and Silk's experiments and this probably accounts for the difference in the ratios.

Summary

1. Succinyldicholine is unstable in alkaline solutions, the rate of destruction increasing with increasing alkalinity. The reaction is of the first order, and at *p*H 9.5 there is 50% loss of activity in just under 3 hr.

2. Mixed with thiopentone, succinyldicholine is rapidly hydrolysed, with 50% loss of activity in 90 min. There is apparently a reaction between the succinyldicholine and the thiopentone, since the decay curve shows it to be a second-order reaction.

3. Succinyldicholine is unstable in solution at temperatures above 40° C. At 100° C. there is 50% loss of activity in 30 min. The reaction is of the first order.

4. *In vitro,* succinyldicholine is hydrolysed by pseudo-cholinesterase but not by true cholinesterase or eserine-inhibited pseudo-cholinesterase.

5. *In vivo,* destruction of true cholinesterase by injection of 284C51 (a specific inhibitor of true cholinesterase) does not alter the response to succinyldicholine, whereas inhibition of the pseudo-cholinesterase by eserine considerably sensitizes the animal to succinyldicholine. Since maximum sensitization of a muscle is not achieved until 1 hr. after the eserine has been given intravenously, and since in rabbits there was no correlation between plasma pseudo-cholinesterase level and sensitivity of a muscle to succinyldicholine, it is suggested that factors other than plasma pseudo-cholinesterase concentration assist in determining the final effect of succinyldicholine on the muscle.

6. Succinylmonocholine has been compared with succinyldicholine and found to be qualitatively similar in its actions but considerably less potent.

I wish to acknowledge the help and advice given by Dr. A. C. White, and the competent technical assistance of Miss L. S. Brooks and Messrs. F. Huggins and D. J. Tulett.

This paper was in part communicated to the meeting of the British Pharmacological Society on January 3, 1953.

ADDENDUM

PREPARATION OF SUCCINYLMONOCHOLINE ESTER SALTS

By Tudor S. G. Jones and R. H. Pain

The preparation of succinylmonocholine ester salts has been recently described by Phillips (1953), but before this a method was tried independently in order to provide material for the above and other work. Phillips found two methods to give satisfactory yields of salts of the ester: (1) esterification of 2-dimethylaminoethanol with acetic anhydride, followed by quaternation with methyl iodide, (2) direct esterification of choline iodide with acetic anhydride, in a melt at 140° C. A third attempt by partial hydrolysis of the di-ester iodide with alkali gave only some unchanged di-ester, succinic acid and sodium iodide. Our experiments, including examination of the products by paper chromatography, are in complete agreement with this last finding. The method of preparation adopted by us involved the discovery of a solvent—nitromethane—which dissolved both choline chloride and succinic anhydride.

Preparation of Succinylmonocholine Ester Chloride.—Choline chloride (7 g.) dried over KOH *in vacuo* and succinic anhydride (10 g., 2 equiv.) were dissolved in nitromethane (250 ml.) and refluxed for 3 hr. The solvent was removed by distillation. The residue was extracted under reflux successively with 350 ml. and 200 ml. of anhydrous ethyl ether and finally washed with dry ether. The product was dissolved in a minimum of hot ethanol and ether added to incipient crystallization. On cooling, a fine crystalline precipitate (10.7 g.) was obtained, filtered off and washed with dry ether. On recrystallization the yield was 9.9 g., m.p. 150–151°. It is readily recrystallized from ethanol. (Found: C, 45.1; H, 7.2; N, 5.9; Cl, 14.80. $C_9H_{18}NO_4Cl$ requires C, 45.1; H, 7.5; N, 5.9; Cl, 14.80%.)

Preparation of Succinylmonocholine Ester Perchlorate.—The chloride (1.7 g.) was dissolved in water and an equivalent volume of silver perchlorate solution added. After boiling to coagulate the AgCl, which was removed by filtration, the solvent was evaporated under reduced pressure and the residual oil dried azeotropically with ethanol and benzene. Crystalline material was obtained from strong ethanol solution by seeding a strongly cooled solution, m.p. 116–117°. (Found: C, 35.6; H, 5.8; N, 4.8. $C_9H_{18}NO_8Cl$ requires C, 35.6; H, 6.0; N, 4.6%.)

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THE ABSORPTION, TOXICITY AND EXPERIMENTAL ANTITUBERCULOUS ACTION OF 5-AMINO-7-METHYL-1:2:4:6-TETRA-AZAINDENE

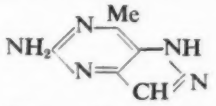
BY

J. FRANCIS,* A. R. MARTIN, F. L. ROSE, AND A. SPINKS

From Imperial Chemical Industries, Limited, Hexagon House, Blackley, Manchester, 9

(RECEIVED JULY 7, 1954)

During the course of routine testing of compounds in the chick embryo, Francis and Hoggarth (1953) detected antituberculous activity in the purine analogue 7438, 5-amino-7-methyl-1:2:4:6-tetra-azaindene. The chemistry of this



substance, together with that of many related compounds which had little or no antituberculous action, has been described by Rose (1952). The tetra-azaindene was next tested in the mouse and found to be of the same order of activity as streptomycin when treatment commenced at the time of infection. This led to an investigation of its activity in guinea-pigs, dogs and monkeys, and to toxicological studies preparatory to a trial in clinical tuberculosis. Although such a trial was not subsequently undertaken, the observation of marked therapeutic activity in experimental infections of this novel chemical type is felt to justify publication of the results as they now stand.

METHODS

The drug exhibits a very strong fluorescence in ultraviolet light. It was estimated in blood by precipitating the proteins with trichloroacetic acid and reading the fluorescence of the filtrate in the Coleman fluorimeter. It may also be estimated spectrophotometrically.

The antituberculous action of the drug was assessed in mice by the methods previously described (Martin, 1946; Hoggarth and Martin, 1950). The method of assessing lesions in guinea-pigs was that described by Francis, Spinks, and Stewart (1950).

For the intracerebral inoculation of guinea-pigs a 20 gauge needle and 0.1 ml. of inoculum were used. A small hole was made in the skull just to one side of the midline, on a line joining the right eye and left ear.

RESULTS

Absorption and Toxicity in Various Species

Mice.—When a dose of 250 mg./kg. of 7438 was given by stomach tube, the drug was well absorbed, reaching a maximum concentration in the blood of 5–6 mg./100 ml. Its rate of clearance from the blood was similar to that of sulphanilamide, the concentration falling to 1–2 mg./100 ml. about 9 hr. after dosing.

The drug was of low toxicity to mice. Animals given doses of 300 mg./kg. twice daily *per os* for 6 weeks gained weight at the same rate as untreated controls, but similar doses of 400 mg./kg. caused the death of 6 out of 12 mice in one week. Doses of 500 mg./kg. killed 8 out of 12 mice in two days. Mice kept on powdered food containing 0.25% of 7438 (i.e. intake approximately 0.5 g./kg./day) for 6 months gained weight at exactly the same rate as control mice.

Guinea-pigs.—Guinea-pigs increased normally in weight when dosed twice daily with 50 mg. 7438/kg., but not when dosed with 75 or 100 mg./kg. They gained in weight when given 0.2% but not 0.4% 7438 in powdered food. One hr. after dosing with 100 mg./kg. three animals had the following blood concentrations: 1.75, 4.31 and 5.27 mg./100 ml.; 16 hr. after dosing the concentrations were 0.71, 1.6 and 3.3 mg./100 ml. Groups of four guinea-pigs which had received food containing various concentrations of 7438 for 19 days had the following mean concentrations of drug in the blood: from 0.1% of drug in the food, 0.2 mg./100 ml.; 0.2%, 0.7 mg./100 ml.; and 0.4%, 1.3 mg./100 ml.

Dogs.—Four dogs were given single oral doses of 7438. The concentrations of drug in the blood after the administration of 100 mg./kg. rose to

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maxima ranging between 2.5 and 5.5 mg./100 ml. after 2–3 hr., and fell to about half these values 7 hr. after dosing. The concentration of 7438 in the cerebrospinal fluids of the dogs was about two-thirds of the concentration in the blood, showing that the drug passed freely into this medium.

Three dogs were given two doses of 7438 daily. One dog was given 100 mg./kg. morning and evening for one week, a second dog 75 and 100 mg., and a third dog 50 and 75 mg. The animal receiving the largest doses vomited once, but showed no other signs of toxic effects, and it was therefore decided to give morning doses of 50 mg. and evening doses of 100 mg./kg. to tuberculous dogs. After four days this dose was increased to 100 mg./kg. twice daily, but it caused vomiting in two dogs, one of which died. The liver was yellow and there was extensive central necrosis of the lobules There was also some necrosis of the epithelium of the renal tubules. After 22 days' treatment the dose of 7438 was stabilized at 30 mg./kg. twice daily, which maintained blood concentrations between 1 and 4 mg./100 ml. The dogs were killed after about 15 weeks' treatment and, apart from the tuberculous lesions, the dogs treated with 7438 showed some cirrhosis of the liver and vacuolation of liver cells (sections were not stained for fat).

Monkeys.—Two monkeys, recently purchased, which had a positive reaction to tuberculin, were given a single dose of 7438. The first received 200 mg. and the second 100 mg./kg. The latter died suddenly whilst the third hourly blood sample was being taken; post-mortem examination revealed extensive tuberculous broncho-pneumonia. The concentrations of 7438 in the blood of the former 1, 2, 3, 5, and 24 hr. after dosing were 3.2, 3.4, 3.6, 5.6 and 2.5 mg./100 ml., respectively. Another monkey was dosed with 30 mg./kg. twice daily for 60 days. During this period the weight of the animal increased from 3.2 to 3.6 kg. Blood concentrations were determined on five occasions during the period of dosing and averaged approximately 5 mg./100 ml. The concentration of blood urea on the 29th, 42nd and 56th day was 71, 65 and 40 mg./100 ml., respectively. Blood counts and haemoglobin estimations, done at the same times as the 7438 estimations, revealed no significant changes.

Action on Tuberculosis

7438 had little action on tubercle bacilli *in vitro*. Some growth occurred in Dubos' medium containing 1:1,000 of the substance, and growth was normal in the presence of 1:27,000.

Mice.—When given by mouth to mice infected intravenously with "human" type tubercle bacilli, the tetra-azaindene produced a marked increase in mean survival time. For this reason it was used for a considerable period in these laboratories as a standard or reference substance, being given for the first 14 days after infection. It was also active when given subcutaneously from 1–5 or from 8–12 days after infection. The results of a number of experiments are shown in Table I. These effects listed are of the same order as those produced by the subcutaneous administration of streptomycin, 1 mg./20 g. twice daily for the same limited period.

TABLE I

INCREASE IN MEAN SURVIVAL TIME (M.S.T.), IN DAYS, OF GROUPS OF 10 MICE INFECTED I.V. WITH *M. TUBERCULOSIS* AND TREATED WITH 7438 FOR THE FIRST 14 DAYS

Drug Given *per os* Twice Daily. Each Dose, mg./kg.			Drug Given S.C. Once Daily in Oil. Dose, mg./kg.	Drug Given in Food. Total Daily Intake, mg./kg.		
50	150	250	500	200	500	1,000
0	4·7 5·7 5·7	3·8, 2·8 4·1, 6·0 4·7, 2·2	7·7	3·9	4·4, 3·2 5·1, 2·1 4·8, 5·0 5·7, 4·4 3·8, 4·3	7·3

M.S.T. of control groups 19·2–23·5 days. Increase of M.S.T. required for significance ($p = 0.05$), 1·2–2·3 days.

When administration of 7438 (500 mg./kg. per day in food) was continued from the time of infection (with 0.75 mg. of bacteria) up to 180 days, only one specific death occurred (on the 150th day) in a group of 15 mice. The remainder were alive and apparently in excellent condition (mean weight 43 g.) when the experiment was terminated on the 210th day, but on examination *post mortem* they were found to have some tuberculous lesions in the lungs. It appeared that the mice might have died from a tuberculous infection after a very long time. Streptomycin was not included in this experiment, but 80% of a group treated with *p*-ethylsulphonyl benzaldehyde thiosemicarbazone (8388) (500 mg./kg. per day in food) were dead at the end of the experiment.

Treatment with 7438 (500 mg./kg./day in food) of a group of mice infected intravenously with 0.02 mg. of organisms was included in Expt. No. 241 (Hoggarth and Martin, 1950), and gave results very similar to those given by 8388, i.e. results much less striking than those of streptomycin at a dose of 37.5 mg./kg. twice daily. The effect of 7438 was therefore much less when it was used

to treat an established infection than when it was given from the time of infection onwards.

Guinea-pigs.—Five groups of seven guinea-pigs were inoculated subcutaneously with 0.1 mg. of human tubercle bacilli (Weybridge C) and given medicated food or dosed as shown in Table II. Only one control guinea-pig had died from tuberculosis, after 42 days, when the rest were killed and the severity of the lesions assessed. It will be seen from Table II that 7438 had about the same effect when given in the food as by mouth and that it was considerably less effective than streptomycin but more effective than 4:4′diaminodiphenyl sulphone.

TABLE II

EFFECT OF VARIOUS DRUGS ON TUBERCULOSIS IN GUINEA-PIGS INJECTED SUBCUTANEOUSLY WITH 0.1 MG. "HUMAN" TUBERCLE BACILLI (WEYBRIDGE C); TREATMENT WAS BEGUN IMMEDIATELY

Treatment	No. of Animals per Group*	Average Score of Lesions	Average Weight of Spleen g.
50 mg./kg. streptomycin twice daily	7	0·23	0·96
50 mg./kg. 7438 orally twice daily	5	1·0	1·8
0·25% 7438 in food	6	1·0	1·2
0·3% 4:4′diaminodiphenyl sulphone in food	4	2·3	5·0†
Controls	4	3·8	4·0

* Some non-specific deaths occurred.
† Sulphone specifically increases the size of the spleen.

The next experiment in guinea-pigs was one already described (except for the result with 7438) by Francis, Spinks, and Stewart (1950). Briefly, animals were injected intramuscularly with 0.005 mg. of human tubercle bacilli (Weybridge C) and 38 days later they were weighed. Six guinea-pigs were killed and the extent of the lesions assessed; the others divided into groups and treatment commenced. The guinea-pigs were in rather poor condition when treatment began and the drugs caused only a slight prolongation of life, but 7438 and streptomycin were placed in the same order of activity on the basis of survival time and the assessment of lesions (Table III).

Action of 7438 and Streptomycin on Tuberculosis Produced by Intracerebral Injection.—Thirty guinea-pigs were divided into three equal groups. One group was given food containing 0.25% 7438 and a second group streptomycin 20 mg./kg. twice daily. The following day all guinea-pigs were injected intracerebrally with 0.01 mg. of human tubercle bacilli (Weybridge C) and treatment was continued. The control animals survived for an average period of 19 days and those receiving streptomycin for 30.5 days. The 7438 treated animals survived for an average period of only 16 days.

TABLE III

EFFECT OF VARIOUS DRUGS ON TUBERCULOSIS IN GUINEA-PIGS INJECTED INTRAMUSCULARLY WITH 0.005 MG. "HUMAN" BACILLI (WEYBRIDGE C); TREATMENT WAS BEGUN 38 DAYS LATER

Treatment	No. of Animals per Group	Average Time in Days from Commencement of Treatment to Death	Average Score of Lesions
0·25% 7438 in food ..	14	19·7	2·5
0·25% 8388 „ „ ..	14	21·2	2·0
20 mg./kg. streptomycin twice daily	13	23·3	1·6
Controls	14	17·9	3·7
Animals killed 38 days after infection (when treatment of others began)	6	—	2·6

DISCUSSION

In guinea-pigs, dogs, and monkeys 7438 in single oral doses of 100 mg./kg. produced maximum blood concentration of about 4 mg./100 ml. after three to five hours. The drug was more persistent in monkeys than in dogs and produced higher concentrations on repeated dosing. Mice tolerated oral doses of 250 mg./kg. twice daily, but guinea-pigs, dogs, and monkeys tolerated only about one-tenth of this.

The results presented in this paper, together with those recorded by Hoggarth and Martin (1950), indicate that, although 7438 has a very good action if mice are treated immediately after infection, the drug is considerably less active than streptoymcin, and probably less active than *p*-ethylsulphonylbenzaldehyde thiosemicarbazone (8388), against established tuberculosis in mice and guinea-pigs. Francis (unpublished) obtained essentially similar results in dogs and monkeys, and unless 7438 is considerably less toxic to man than to other species it is unlikely to be of value in the treatment of human tuberculosis.

SUMMARY

1. 5-Amino-7-methyl-1:2:4:6-tetra-azaindene (7438) was well absorbed when given orally to mice, guinea-pigs, dogs and monkeys; it was fairly persistent in the blood and passed freely into the cerebrospinal fluid of dogs.

2. Mice tolerated repeated oral doses of 250 mg./kg. of the drug, but guinea-pigs, dogs and monkeys tolerated repeated doses of only 25–50 mg./kg. Doses of 100 mg./kg. in dogs produced necrosis of the liver and death.

3. At its maximum tolerated doses, 7438 was of the same order of effectiveness as *p*-ethylsulphonylbenzaldehyde thiosemicarbazone against established tuberculosis in mice. The drug was considerably less effective than streptomycin against established tuberculosis in mice and guinea-pigs. Against cerebral tuberculosis in guinea-pigs 7438 had no effect, whereas streptomycin given systemically caused a 50% increase in the mean survival time.

4. It is concluded that 7438 is unlikely to be useful in the treatment of human tuberculosis.

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THE EFFECTS OF ANTICHOLINESTERASES ON HUMORAL TRANSMISSION IN THE SUBMAXILLARY GLAND

BY

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The purpose of the investigation was to study the effect of anticholinesterases on the secretion of saliva by the submaxillary gland, and especially the spontaneous secretion provoked by those agents. The humoral transmitter of parasympathetic stimuli for salivary secretion has been shown to be acetylcholine (Beznák, 1932; Gibbs and Szelöczey, 1932), and that for sympathetic stimuli adrenaline (or noradrenaline) (Cattell, Wolff and Clark, 1934). There are definite but unexplained inter-relations between these two forms of nerve or humoral stimuli.

METHODS

Cats and dogs were used. Anaesthetics were pentobarbitone sodium ("Nembutal"), intraperitoneal or intravenous; chloralose, intravenous; urethane, intravenous; allobarbitone ("Dial")-urethane, intraperitoneal.

Both submaxillary ducts were usually cannulated, the left gland being used for the experimental modification and the right as a control. The saliva secreted was recorded by drop recorders, electronically operated, the jets delivering drops of 0.01 ml. for cats and 0.03 or 0.05 ml. for dogs. In order to obviate variation in drop size or viscosity, each saliva delivery tube was led to a bulb of about 200 ml. capacity filled with solution containing about 0.5% NaCl and 0.1% Na_2CO_3; the saliva entered at the bottom, and the saline solution was displaced through a tube at the top.

The chorda tympani and cervical sympathetic were prepared for stimulation when required; in the earlier experiments stimulation was effected by an Attree stimulator, at an appropriate voltage to give a submaximal stimulus, pulse width being usually 0.1 msec., and frequency 10 cycles/sec. (Wills, 1941). In the later experiments this stimulator was replaced by an inductorium having a mains transformer of 5V, in the primary. Electrodes were either a simple form of fluid electrode (Garry and Wishart, 1951), or plain double stainless steel electrodes applied to the nerve. Arterial pressures were recorded from the femoral artery with a mercury manometer.

Injections of chemical substances into the circulation were sometimes made intravenously into the femoral vein, but, more commonly, intra-arterially (at constant speed and volume) by a close arterial injection so as to be more or less localized to the district of one submaxillary gland. For this purpose the lingual artery was chosen, using a valved cannula of small capacity. In some of the earlier experiments we tried to limit the blood supply closely to the submaxillary gland by tying off all branches beyond the facial, but later this practice was often abandoned, and in consequence the close arterial injection did not affect the submaxillary gland alone; other regions affected were the parotid gland, the external ear, parts of the scalp, and some muscles of the face and pharynx. So far as we were able to judge, these areas of escape did not materially affect our results, except that larger doses were needed.

In some experiments the cholinesterase content of both glands was determined at the end, the right gland, which had not been injected, serving as a control: in other experiments the control gland was removed early in the experiment, and before any injections had been given. The glands were first frozen, cut up with a microtome, and then homogenized with a Potter-Elvehjem homogenizer. The activity was determined against acetylcholine, 0.015M, acetyl-β-methylcholine, 0.03M, and butyrylcholine, 0.03M, or benzoylcholine, 0.015M. Blood samples were also tested before and at the end of the experiment against acetylcholine, 0.015M.

In spite of every care in the homogenization of the tissue, there was some irregularity in the results, some of which were discarded.

As anticholinesterases we tried a variety of compounds, one of our aims being to inhibit, preferentially in one gland and not in the other, either the true or the pseudo-cholinesterase. In this we were more successful when using the pseudo-inhibitors than when using those which specifically inhibited the true cholinesterase, probably for the reason that the latter did not remain so firmly fixed at the sites of first presentation.

As specific inhibitors of true cholinesterase we used *N*-*p*-chlorophenyl-*N*-methyl carbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu 1250) (Hawkins and Mendel, 1949), and 1:5-bis(4-allyldimethylammoniumphenyl)-*n*-pentan-3-one dibromide

* The survival operations were performed by C.L.E.

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(BW 284C51) (Austin and Berry, 1953). We found the Nu 1250 the better, because of its slower reversibility.

For specific inhibition of the pseudo-enzyme we used tetra-*iso*propyl pyrophosphoramide ("Isoompa"), bis(mono*iso*propylamine)phosphinic fluoride ("Isopestox"; "Mipafox"), and di*iso*propyl phosphorofluoridate (DFP).

Inhibitors also used, and which acted on both types, were eserine, tetraethyl pyrophosphate (TEPP), *iso*propyl methylphosphonofluoridate ("Sarin"), ethyl *NN*-dimethylphosphoramidocyanidate ("Tabun"), cyclohexyl methylphosphonofluoridate (called "CHX" for short).

Results

Response of the Submaxillary Gland to Transmitters

Close arterial injections of ACh evoke a secretion, the threshold dose varying considerably, according to the extent to which the substance is delivered to situations other than the glands concerned, to the rate of delivery of the injection into the blood stream, and to the state of the local circulation. When the arterial delivery area is restricted solely to the submaxillary (and sublingual) glands, doses as small as 0.01 μg. were found by Gibbs (1935) to be effective, and to be about as effective as (presumably maximal) chorda stimulation for 5 sec. In our experiments, in which the arterial supply was usually not restricted exclusively to the submaxillary and sublingual glands, we have used doses varying from 1 to 50 μg., and obtained responses usually over the threshold. The responses of the glands on the two sides were normally about equal, but there was a good deal of difference from one animal to another. Some glands seemed to be remarkably insensitive, and this seemed to be especially so with very large and old cats; no explanation is offered for this, but it was noted that in these instances the response to chorda stimulation and to adrenaline was normal.

Response to the less fugitive adrenaline could be obtained even by intravenous injection, and this method of administration has been regularly used by Emmelin (1953) in his recent studies. On arterial injection we usually got a small response to 5 to 10 μg. of adrenaline (Fig. 1). Noradrenaline was at most only about one-third as effective as adrenaline; this difference was reduced, and both responses increased, if cocaine was previously given. It may be, therefore, that the noradrenaline was less effective because of its more rapid destruction by amine oxidase; we did not pursue this point further. But it is in any case not due to the more intense vasoconstriction produced in the gland by the noradrenaline, because adrenaline injected at the peak of the rise of blood pressure caused by noradrenaline is as effective as when given alone (Fig. 1). Atropine in large doses to cats abolishes or reduces the response to adrenaline, a fact already well known.

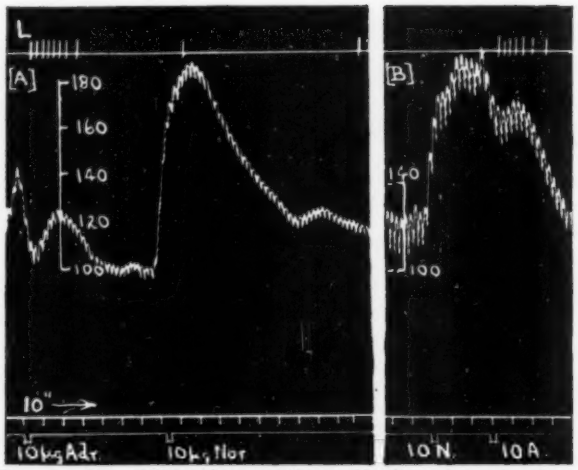

FIG. 1.—Cat. Allobarbitone-urethane. Tracings, in order from above down: top (L) drops of saliva from left Wharton's duct; second, femoral artery pressure; third, time, 10 sec.; bottom, signals. (A): 10 μg. adrenaline, then 10 μg. noradrenaline. (B): 10 μg. noradrenaline, and, at crest of blood pressure, 10 μg. adrenaline. In this and subsequent records, all injections are close arterial injections except where otherwise stated.

General Effect of Anticholinesterases

The effects of the intra-arterial injection of anticholinesterases differed according to whether the dose was small or large, and to the degree of reversibility of the effect on cholinesterase; there was also a broad difference in the effects of different anticholinesterases, according to their actions respectively on true and on pseudo-cholinesterase. Owing, however, to the fact that specific inhibitors of true cholinesterase are generally reversible, while those which specifically inhibit the pseudo enzyme tend to be irreversible, there was considerable overlapping in the types of action.

When the dose was small, but large enough to produce some effect, this consisted of potentiation of the action of ACh and of chorda stimulation. If the inhibition was reversible, these effects might also appear later on in the other gland, but, even with an easily reversible agent like eserine, the effect was often localized for a considerable time (15–30 min.). In that case, or when the reversibility was only slowly affected, as with TEPP or Nu 1250, the state of affairs seen in Fig. 2 appears. It is seen that the potentiation is confined to the initially poisoned side for at least 15 min., and that stimulation of the chorda tympani, or administration of ACh, on the poisoned side, causes secretion

(potentiated) on that side only. It was sometimes seen also that ACh administered to the unpoisoned side caused secretion from both glands, and that chorda stimulation or ACh applied to the poisoned side caused a fall of blood pressure much greater than that caused by similar treatment of the unpoisoned side.

With small doses of reversible inhibitors, the effects of potentiation of the action of ACh and of chorda stimulation were more clearly seen, but for the specific pseudo-inhibitors the doses necessary to produce effects were (taking the relative toxicities into account) sometimes rather large when compared with those of the reversible (true) inhibitors which produced similar effects.

As the dose of anticholinesterase was increased the effects described above were all intensified. The effects of ACh injection and of chorda stimulation were greatly augmented, and, especially after the dosage with a reversible inhibitor, were ultimately seen with both glands; ACh given intra-arterially to one gland, or stimulation of the chorda to one gland, were both often followed some seconds later by a fall of blood pressure, as was noted by Babkin, Gibbs and Wolff (1932), and by secretion of the other gland.

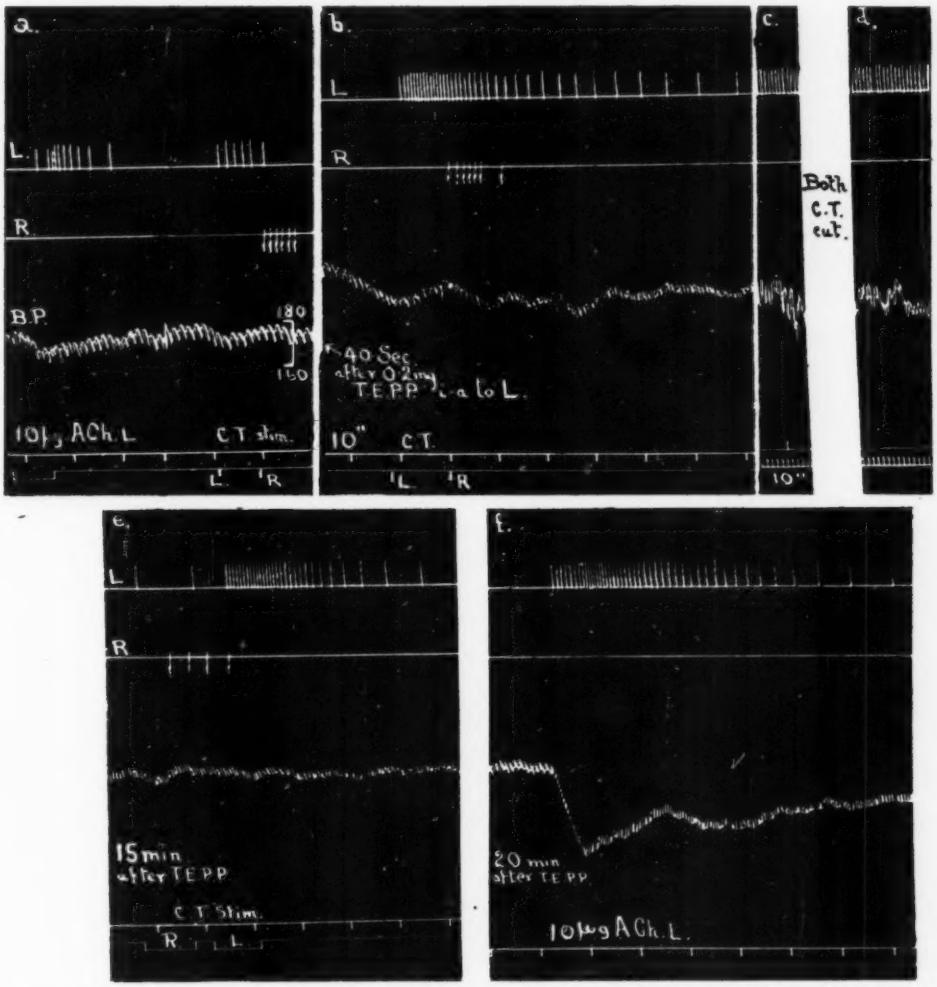

FIG. 2.—Dog. Allobarbitone-urethane. Records from above down, as before, except for (c) and (d) in which time trace and signal were transposed. Injections into left lingual artery. Stimuli 10/sec. 1 msec. (a): effect of 10 μg. ACh and of 10 sec. stimulation of left and of right chorda tympani. (b): begins 40 sec. after dose of 0.2 mg. TEPP i.a. to L; effect of 10 sec. stimulation of L and of R chorda. (c): 1 min. after (b) on slow drum to show steady flow from L gland. Between (c) and (d), also on slow drum, both chordas cut. (e): 15 min. after the initial dose of TEPP, potentiation of effect of 10 sec. stimulation of chorda is still only seen on L. (f): 5 min. later, spontaneous flow has stopped; 10 μg. ACh given.

At a certain further stage of poisoning it was found that stimulation of the gland, either by ACh or *via* the chorda, not only produced prompt and abundant flow of saliva, but also a steady and slowly dwindling after-flow (Fig. 2b). This after-flow was usually confined to the side stimulated, and might last for several minutes, the other gland remaining unaffected ; but sometimes, after a time-lag of about a minute, the second gland also began a slow rate of secretion which might even outlast that of the first affected gland. These phenomena of spread were most conspicuous with the reversible and true inhibitors.

With further dosage, and in every instance in which the dosage was carried to the point of producing symptoms of general poisoning, such as muscular fasciculations, a "spontaneous" secretion of saliva began in the initially poisoned gland ; sometimes only the initially poisoned gland gave a spontaneous flow, but often, especially with reversible inhibitors, the other gland followed ½–2 min. later (Fig. 3c). In some instances the gland remained quiescent until the chorda was stimulated or a dose of ACh was given, when the first brisk response started off a prolonged spontaneous secretion (Fig. 2). The spontaneous secretion, once established, sometimes continued indefinitely, and was very abundant, but at other times, where the dose was only marginal for this effect, it slowly faded out, but could be revived again by a further dose of the anticholinesterase. With eserine the spontaneous flow soon slackened off, and larger and larger doses were required to maintain it.

These results can probably be satisfactorily explained in terms of the ease of reversibility of the poisoning of the cholinesterase, and of the site and amount of the dose of the drug given. If the dose is small, not too rapidly injected, and the action irreversible, then the anti-ChE is fixed in the tissues of the injected field and in some of the blood which carried it there, but being immobilized will have but little if any effect in any other parts of the body. When the chorda of the injected side is stimulated, or when ACh is injected into that side, the stimulating effect will be intensified and prolonged because of the inactivation of the ChE in the gland or in its ganglia. For the same reason, and because the ACh set free by stimulation, or injected into the gland arteries, is not destroyed, it is passed on into the general circulation and causes a fall of arterial blood pressure. In our experience, the potentiation of the action of ACh and of chorda stimulation appear together, and in somewhat the same proportion, in which we are in agreement with Gibbs (1935).

With the reversible anticholinesterases, the drug, though fixed, is less firmly held in the tissues, and so is sooner or later washed out by the circulating blood and distributed all over the body. After an interval of time, therefore, if the dose be very small, the gland initially dosed will cease to show any effect, such as a potentiation of stimulation, nor, because of the smallness of the dose, will there be any effect on the other gland. Nevertheless we have been surprised by the relative tenacity with which even an easily reversible inhibitor like eserine is held in the gland. If the dose is a large enough one, however, the gland on the other side will sooner or later be affected, and will show the potentiation or even the spontaneous flow of saliva.

Causation of the Spontaneous Flow

The spontaneous flow of saliva which follows upon the administration of a sufficient dose of any of the anticholinesterases might be attributed to one or more of a number of causes, such as:

(i) To the presence of ACh, formed elsewhere, and accumulating in the circulating blood.

(ii) To a central action of the drug upon the salivary centres in the medulla.

(iii) To an action upon the ganglion cells on the course of the chorda fibres.

(iv) To an action upon cholinesterase at some site near to the secreting cells, e.g., in the parasympathetic nerve endings or in the gland cells themselves.

(v) To an action of the drug, other than as an anticholinesterase, upon the gland cells.

The first possibility is probably eliminated, at any rate as a main cause, in view of the fact, observed by Emmelin and Muren (1950), and which we have confirmed, that there is a continuous secretion of saliva from a gland perfused with eserinized blood or plasma ; moreover, the onset of secretion following the intra-arterial injection of a large dose of anticholinesterase would seem too prompt to admit of this explanation. But after large doses of anti-ChE it is possible that circulating ACh may later on be a minor contributory factor, because there is sometimes seen a slight build-up of secretion rate for some minutes after an initial injection.

That a central action of eserine affected blood flow and secretion of the glands was claimed by Heidenhain (1872), but this explanation is unquestionably inadmissible in these experiments, since the secretion rate is unaffected by section of all the nerves to the gland (Fig. 2d).

The third possibility, viz., that there might be some action on the relay ganglia on the course of

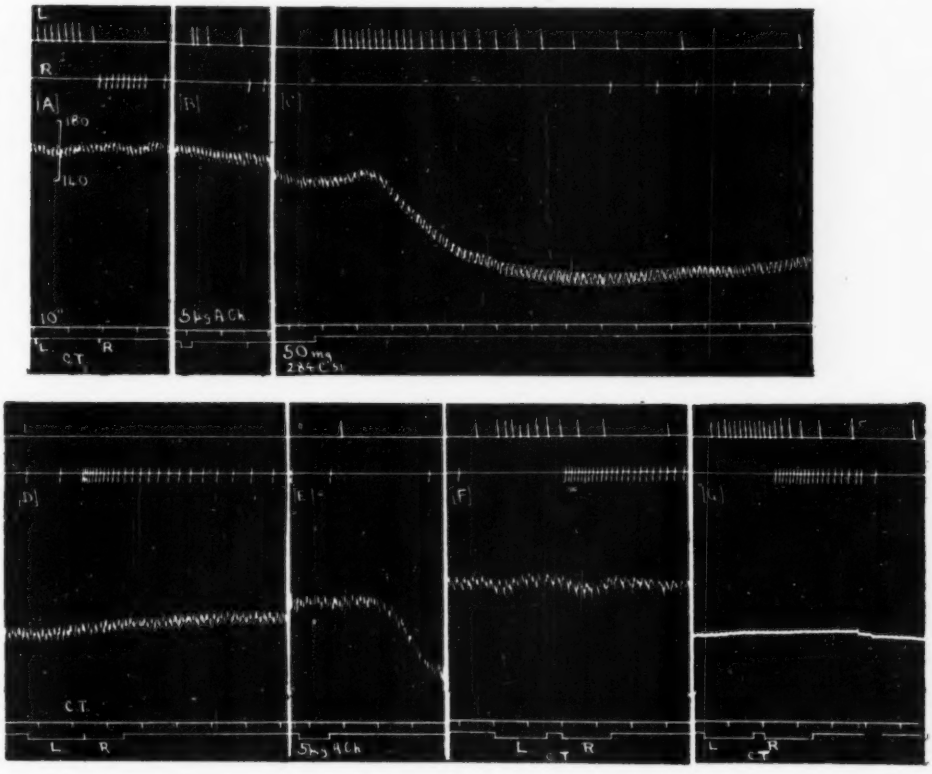

FIG. 3.—Dog. Allobarbitone-urethane. Injections into L lingual artery. (A): stimulation of L and R chorda tympani. (B): 5 μg. ACh. (C): 50 mg. 284C51. (D): stimulation of L chorda ineffective, that of R potentiated. (E): 5 μg. ACh also almost inactive. (F): slow spontaneous flow has returned to L, and was slightly potentiated by chorda stimulation, some 5 min. after (E). (G): 10 min. after F, full potentiation restored.

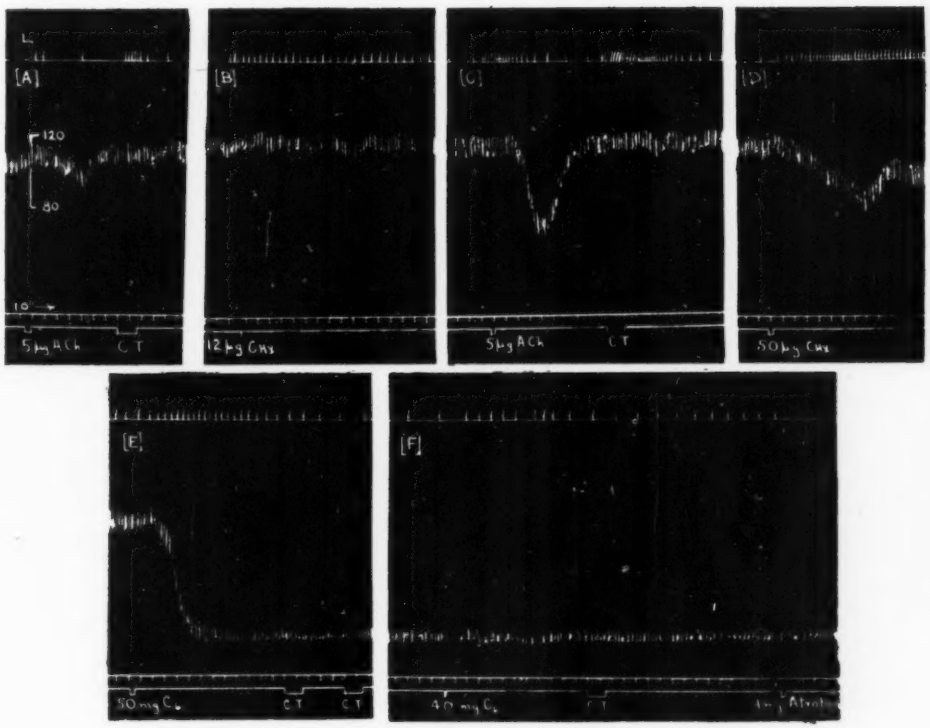

FIG. 4.—Dog. Allobarbitone-urethane. L, submaxillary duct; blood pressure; time 10 sec.; signals. (A): test dose of 5 μg. ACh to L and stimulation of L chorda 10/sec. (B): 12 μg. "CHX" to L artery. (C): 5 μg. ACh and stimulate L chorda. (D): 50 μg. "CHX" starts rapid spontaneous flow. (E): 50 mg. C6 slows but does not stop flow; stimulation of c.t. now ineffective. (F): 40 mg. C6 does not stop flow; 1 mg. atropine does so. Note C6 at first accelerates flow.

the chorda, is less easily appraised. It could, for instance, be claimed that in the perfused gland the scattered ganglion cells contained in it had also been affected by the drug. But, on the other hand, the administration of even large doses of ganglion-blocking agents, such as hexamethonium (C6), tubocurarine, M and B 2050, or nicotine, failed to do more than first to accelerate, and then, often only temporarily, slow down the flow (Fig. 4), except in cases where there was a catastrophic fall in blood pressure. The action of tubocurarine differed from that of the other agents, inasmuch as large doses given beforehand did prevent the spontaneous flow, whereas when given after the flow had been established only slowing occurred. Thus, in Fig. 13, the spontaneous flow due to 1.4 mg. of Nu 1250 to each gland was only slowed by a total of 40 mg. of tubocurarine, but in another experiment a preliminary dose of 12.5 mg. tubocurarine prevented spontaneous secretion when 7.5 mg. Nu 1250 was given to one gland alone. The dose of Nu 1250 usually needed to produce spontaneous secretion was from 0.05 to 0.5 mg. intra-arterially. After these heavy doses of blocking agents the chorda was always found to be blocked (Fig. 4), but ACh to be still effective. In one experiment the chorda and the hilum of the gland were painted with 10% nicotine in order to block the ganglia preferentially, but with no effect on the spontaneous flow, caused by 275 μg. of Nu 1250.

The fourth possibility, that the essential action is upon cholinesterase in or near to the secreting cells, is what would be expected. We should suppose this cholinesterase to be situated near to the parasympathetic nerve endings, though there is much uncertainty as to the structural relation of these to the secreting cells. Further, the humoral transmission hypothesis would lead us to expect that, in the normal functioning of the secretory cells in response to chorda impulses, ACh would be liberated at or near to the nerve endings. Both essential constituents are present, for the gland contains plenty of cholinesterase, and Chang and Gaddum (1933) have shown that the dog's submaxillary contains from 1.5 to 3.3 μg. ACh/g., while Emmelin and Muren (1950) found the cat's chorda to contain about 1.5 μg./g.

It seems likely that the relation between nerve endings, ganglion cells and effector cells in the salivary glands is similar to that which obtains in the small intestine. Feldberg and Lin (1949) showed that ACh is constantly being set free in the small intestine, and they inferred that it originated either in the nerve endings or in non-nervous structures. As regards the salivary glands, the recorded findings show a discrepancy, for although Beznák (1932) and Gibbs and Szelöczey (1932) could find no ACh in the effluent from perfused glands prior to chorda stimulation, its presence was demonstrated by Henderson and Roepke (1933) and by Emmelin and Muren (1950), the amount being greatly increased by chorda stimulation. The last-named authors found that when curare was first added, chorda stimulation only released about one-third as much ACh, and this amount they thought might have come from preganglionic nerve endings.

If we accept the opinion that ACh is normally set free constantly from some structures (" nerve endings ") peripheral to the parasympathetic ganglion cells, and suppose that cholinesterase is present in the vicinity of liberation, so that under normal conditions the small amounts set free at the endings are not effective in producing secretion, though perhaps keeping the gland in a condition of constant subliminal excitation, then it would be reasonable to admit that after poisoning with an anticholinesterase the small amounts of ACh liberated will accumulate, and ultimately will reach a threshold concentration so that secretion will ensue. Since this flow is not stopped by the action of ganglion-blocking agents, it may be inferred that the source and site of action of the ACh cannot be wholly in the ganglion cells ; but that the flow is slowed by ganglion-blocking agents might be interpreted to mean that part of the secretion originated similarly by the action of ACh spared at ganglionic synapses. This seems unlikely, however, since, so far as we know, ACh is not liberated at synapses except on the arrival of impulses along the presynaptic fibres (Feldberg and Gaddum, 1934), and moreover it has been shown that eserine can rather have a paralysing action on ganglionic transmission (Feldberg and Vartianen, 1935).

We conclude, therefore, that the site of the liberation of the ACh is mainly if not entirely at, or near to, the endings of the parasympathetic nerves ; atropine in quite small doses at once checks the spontaneous secretion, presumably by acting peripherally to that site, and, once this has happened, even enormous doses of anticholinesterase may fail to restart the spontaneous secretion. In fact, when the dose of atropine is adequate, say over 20 μg. intra-arterially, we have been unable to restart the secretion by administering an anticholinesterase, even in doses 8 times larger than those originally needed to start a spontaneous secretion.

The Cholinesterases of the Submaxillary Gland

There is much confusion concerning the nomenclature of the cholinesterases, and we have no wish to add to it; we may admit the existence of two classes of enzyme, which differ not only in their relation to different substrates, but also in their responses to different inhibitors. Enzymes which resemble, say, the cholinesterase of human red cells in being able to hydrolyse ACh and acetyl-β-methylcholine more readily than other esters could reasonably be designated as true cholinesterases if they were also inhibited by those substances which inhibit red-cell cholinesterase. Similarly, enzymes which resemble human serum cholinesterase in rapidly hydrolysing benzoylcholine or butyrylcholine could be provisionally classified as pseudo-cholinesterases if they were found to be inhibited preferentially by the inhibitors of human plasma cholinesterase.

When these two types of enzyme occur together in the same tissue the estimation of their relative amounts is attended with a good deal of uncertainty. But we have made estimations on submaxillary gland tissue, using as substrates ACh, acetyl-β-methylcholine, benzoylcholine and butyrylcholine. The results are given in Table I, which shows that there is considerable variation

TABLE I

CHOLINESTERASE CONTENT OF SUBMAXILLARY GLAND OF CAT AND DOG EXPRESSED AS μl. CO_2/G./HR.

Species	Acetylcholine 0·015M	Benzoylcholine 0·015M	Acetyl-β-methylcholine 0·03M	Butyrylcholine 0·03M
Cat ..	645		282	518
	4,810		2,510	1,390
	6,550		3,200	3,200
	6,475		3,225	2,450
	5,715		2,695	1,428
Mean ..	4,839		2,382	1,797
Dog ..	1,680	—	1,000	1,360
	2,700	—	961	1,682
	1,560	948	1,130	2,415
	1,972	218	1,150	1,660
	3,100	787	2,100	3,580
	3,550	477	2,060	2,498
	477	—	445	111
	1,650	183	1,670	366
	2,840	187	1,917	102
	1,946	154	1,372	—
	842	243	823	—
	2,490	351	2,640	2,230
Mean ..	2,067	394	1,439	1,600

in the amount, but that on the average the cat's gland contains more of the true enzyme than the dog's, and that in both species there is plenty of true cholinesterase, but also some pseudo-cholinesterase.

Relation of True and Pseudo-enzymes to Function

In attempting to link together the types of enzyme with function, we have tried to find whether there was any discoverable relation between the degree of inhibition of one or the other class of enzyme and a clear physiological effect of that inhibition on salivary secretion. For this purpose we chose as indicative of effect the production of spontaneous salivation, and adopted two courses. In some experiments we removed the right gland for control at the start of the experiments, and then, when either secretion or symptoms of general poisoning had been produced by intra-arterial injection of the chosen anticholinesterase into the left gland, this was similary treated, and the change in the cholinesterase content during the experiment determined. In other experiments the injections of drug were made into the left gland, and both glands were removed for assay at the end of the experiment. We had expected that this procedure would be suitable, at all events when the irreversible inhibitors were used, since, from the physiological results, we believe these to produce effects only at the site injected.

In order to test this hypothesis we made an experiment with a radioactive inhibitor (Sarin labelled with ^{32}P), but, although this showed a greater fixation in the gland injected than in the control, most of the radioactivity was in the blood. However, it was disappointingly clear that, despite the clarity of the physiological results, even a supposedly irreversible inhibitor is by no means retained only at the first site, and that much of the inhibitor or its products is carried away by the blood, probably without ever having been fixed in the tissues at all, and although there might be no demonstrable effects produced on other parts of the body.

Table II gives the results of 17 experiments with various anticholinesterases, and is divided into two parts according to whether spontaneous salivation occurred or not. It will be seen that, in all experiments in which no secretion followed, the inhibitor was one which is known to attack the pseudo-enzyme preferentially (and the dose was not large), whereas in all those where the spontaneous secretion was seen the inhibitors were either of the class which attacks both types, such as TEPP, or were specific inhibitors for the true type. It is understood that the degree of specificity *in vivo* is not extremely high, and that if enough of any inhibitor is given both types will be affected, and salivation will ensue. We tried to strike a dose which would inhibit one type very much more

TABLE II

SPONTANEOUS FLOW IN RELATION TO CHOLINESTERASE INHIBITION

Expt.	Species	Drug	Control Out at	% Inhibition of Gland				Blood ACh	Spontaneous Flow
				ACh	MCh	BuCh	BCh		
9	Dog	DFP	End	88	88	—	69	—	No
11	,,	IO	,,	7	10	64	67	—	,,*
15	,,	IP	,,	28	56	87	—	—	,,
16	,,	DFP	Start	42	42	91	81	67	,,
24	Cat	DFP	,,	90	75	98	—	63	,,
26	,,	IO	,,	29	22	99	—	23	,,*
28	,,	IO	,,	20	1	99	—	43	Slow†
30	,,	IO	,,	60	45	73	—	25	Slow‡
			Means	45	42	87	72	44	
10	Dog	284C51	End	28	24	—	36	—	Yes
12	,,	TEPP	,,	74	76	82	66	59	,,
13	,,	TEPP	,,	93	90	86	81	—	,,
14	,,	284C51	,,	62	—	37	—	40	,,
17	,,	CHX	Start	63	77	60	54	85	,,
18	,,	5158	,,	90	85	86	83	100	,,
19	,,	Nu 1250	,,	—	70	35	—	—	,,
20	,,	Tabun	,,	84	77	74	—	46	,,
21	,,	Nu 1250	,,	63	70	26	—	51	,,
			Means	70	71	61	64	63	

IO = Iso-ompa. IP = Isopestox. * No potentiation of ACh or chorda. † 5 mg. cocaine i.v. previously. No potentiation of chorda after IO. ‡ 2 mg. cocaine i.v. previously.

than the other, but it will be seen that this effort was only partly successful. When the right gland was left *in situ* until the end of the experiment, the actual per cent inhibitions of ChE in the left gland must have been greater than appears from our results, especially in the instances in which reversible inhibitors were used—that is, in most of the instances in which the inhibitors acted predominantly on the true enzyme. The figures for whole blood, which were only assayed with ACh, are but little guide in assessing the degree of general poisoning; but if they show anything at all they indicate that this was more general and intense with the reversible inhibitors, which is what would have been expected.

Despite these various shortcomings, however, the mean values have interest, since they suggest that in order to result in a spontaneous flow there must be inhibition of something over 60% of true cholinesterase (with pseudo inhibited to a slightly less extent), whereas an inhibition of 70 to 80% of pseudo-cholinesterase will not result in salivation even if the true esterase is also some 40% inhibited. The generally accepted view that it is the true enzyme which has the more important physiological function is thereby substantiated. But some of the experiments do point to the possibility that the pseudo-enzyme may play a subsidiary part (Table II, Expt. 28), and it is well known that intense salivation is a conspicuous phenomenon in general poisoning with inhibitors such as DFP which preferentially inhibit pseudo-cholinesterase; it is possible, however, that this might be due to circulating ACh. The results of Koelle, Koelle and Friedenwald (1950) and of Burn, Kordik and Mole (1952) on the intestine are of interest in this connexion; they find that a fall in the pseudo-cholinesterase content of the intestine increases the tone of the small intestine, and sensitizes it to ACh. The small intestine in our view resembles the salivary glands in being the site of constant production of ACh, though from what particular sites is again uncertain; but it may well be that the pseudo-cholinesterase in both situations has a general mopping-up function of a subsidiary kind, preventing ACh concentrations from passing a certain limit. That it can only be subsidiary is shown by the fact that relatively large doses of pseudo-inhibitors are necessary even to produce a potentiation of the action of ACh or of chorda tympani stimulation, and in some of the experiments in Table II (11 and 26) despite considerable inhibition of the pseudo-enzyme there was no potentiation. In experiments 28 and 30 small doses of cocaine had been given previous to the iso-ompa, so that the cause of the slow spontaneous secretion is uncertain.

Block Phenomena in the Submaxillary Gland

It is well known that there is an optimal rate of stimulation of the chorda tympani (Willis, 1941) and that very rapid stimulation gives a slower rate of secretion than a slow one. This is an example of the Wedensky inhibition, which is well seen in neuromuscular conduction, where it has been shown (Evans, 1951) to be greatly exaggerated in poisoning by anticholinesterases.

What may be the same phenomenon can also be discerned in the salivary gland (Graham and Stavraky, 1951), though not with the same clarity and precision as at the neuromuscular junction. Fig. 5A is an example, and shows block by ACh during spontaneous flow due to Nu 1250. Sometimes the phenomenon may be seen even in the unpoisoned gland. In Fig. 5B it is seen that immediately following on a dose of 20 μg. ACh to the gland, stimulation of the chorda is much less effective than it was immediately before or about 1½ min. after.

We have seen no instance of "block" being produced by the administration of an anticholinesterase alone, however large the dose. We may interpret this to indicate that the amounts of ACh which are being constantly liberated in the gland are insufficient to cause block, even when the cholinesterase in the gland has been very greatly reduced; no doubt diffusion would account for its

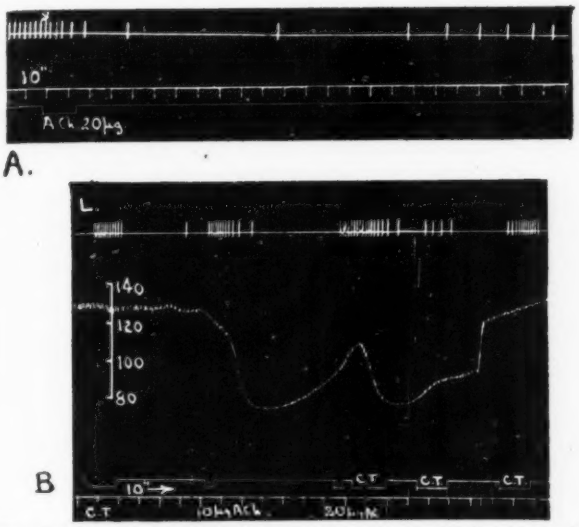

FIG. 5.—Cat. (A): Pentobarbitone. (Chorda cut 10 days before.) Steady flow due to Nu 1250 0.3 mg., 20 min. before. Block by 20 μg. ACh. (B): Allobarbitone-urethane. Second stimulation of chorda blocked and third stimulation partly blocked by a preceding dose of 20 μg. ACh. By the fourth stimulation the effectiveness of the chorda was restored.

failure to accumulate to that extent; but any further access of ACh either from the blood stream when it has been injected, or at tissue sites when the chorda is stimulated, may raise the amount to a level at which block occurs if its destruction is inhibited.

Phenomena of "Augmented Secretion"

It was first shown by Bradford (1888) that the secretory response to stimulation of either the chorda or the sympathetic was augmented if the chorda had been stimulated a few seconds beforehand. (The name "augmented secretion" was given to the phenomenon by Langley in 1889.) Subsequent investigations showed that instances could be found in which stimulation of either nerve disposed the gland to give an augmented secretion when the other nerve was subsequently stimulated (Babkin, 1950).

We have at various times obtained instances of these augmented secretions, and have also found that a dose of ACh could replace a chorda stimulation, and a dose of adrenaline replace sympathetic stimulation; that is to say that sympathetic stimulation or adrenaline gave a larger response when preceded by chorda stimulation (or by a dose of ACh), and *mutatis mutandis* for the other permutations of modes of stimulation. The phenomenon is not always demonstrable, and sometimes it is one permutation which shows it best and sometimes another. Fig. 6 illustrates the augmentation of ACh by adrenaline.

A satisfactory explanation of the phenomenon has never been given, and we only mention it here because it bears on the question of the effect of anticholinesterases on the response to sympathetic stimulation or to adrenaline. In many instances it was found that the response to both these forms of sympathetic stimulation was augmented after poisoning by anticholinesterase. A similar observation was made by Secker (1934), who inferred that ACh acted as the immediate transmitter of sympathetic impulses as well as of the parasympathetic impulses. Feldberg and Guimarãis (1935), however, considered it as merely another instance of the augmented secretion, and this is our explanation. We may suppose that after the poisoning by anti-ChE there is, as after chorda stimulation, sufficient ACh in or near to the gland cells to give an enhanced response when the adrenaline was given, or when the sympathetic was stimulated.

Phenomena of Diminished Secretion

The converse phenomenon of the application of one mode of stimulation resulting in a reduction of the rate of secretion has also been met. One instance of this is that of the occurrence of "block" already referred to.

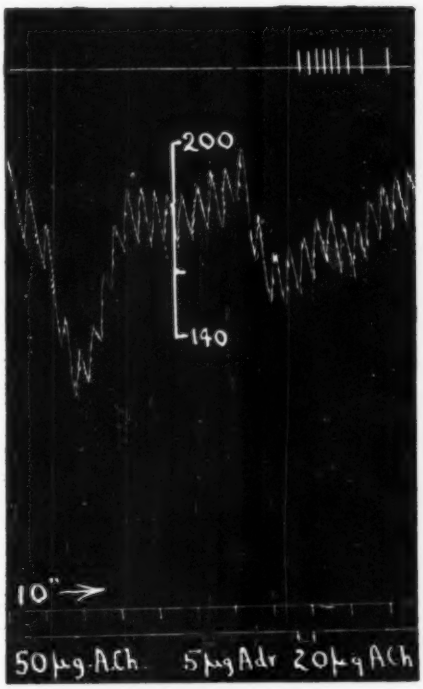

FIG. 6.—Dog. Allobarbitone-urethane. Showing that a gland which was relatively insensitive to ACh (50 μg.) was rendered more sensitive (to 20 μg.) by a dose of adrenaline (5 μg.) which itself had no effect on secretion.

We have, however, from time to time met with another phenomenon, namely, the inhibition by adrenaline or by sympathetic stimulation of a secretion caused by an anticholinesterase. This effect is strikingly seen when a rather large dose (50 μg.) of adrenaline is given intra-arterially during the spontaneous secretion evoked by an anticholinesterase, by pilocarpine, or during chorda stimulation, and is illustrated by Figs. 7, 8, 9 and 10A. Langley (1878) observed that sympathetic stimulation would check the pilocarpine secretion, and a similar result was noticed by Feldberg and Guimarāis (1935) in cats after dosage with eserine, followed by adrenaline. Langley attributed the phenomenon to ischaemia of the glands due to vasoconstriction. But the vascular responses of the gland to adrenaline or to sympathetic stimulation are now believed to be complex. Although Bunch (1900) found a shrinkage in volume of the gland under sympathetic stimulation, he observed that it was followed by a swelling. Cattell *et al.* (1934) found the blood flow to be reduced; but Carlson (1907), Carlson and McLean (1908), and Barcroft and Piper (1912) were all agreed that the constriction was transitory and was followed by a long-lasting and considerable dilatation, so that the blood flow through the gland was mainly increased.

Reduction of the blood supply to the gland has long been known to slow down the rate of secretion, and Carlson and McLean (1908) found that in the dog's parotid it reduced the rate of secretion from pilocarpine, as did also stimulation of the sympathetic. We found that stoppage of the blood supply by clipping off the carotid artery slowed down the spontaneous secretion, but not nearly so dramatically as did an injection of adrenaline (Fig. 8). When a large dose of " Ronicol " (Roche) (β-pyridyl carbinol) was given to produce arterial relaxation, the effect of adrenaline was more transitory (Fig. 9), and, after giving dihydroergotamine, adrenaline caused neither secretion nor slowing of the spontaneous flow; but stoppage of the arterial blood supply soon after this did speedily stop the secretion. This sudden arrest of secretion when the circulation is stopped just after the administration of adrenaline, as compared with the mere slowing which occurred after arrest of circulation in the normal gland, could, we think, be attributed to the fact that adrenaline produces a considerable increase in the oxygen usage of the gland (Barcroft and Piper, 1912), so that the anoxia resulting from ischaemia is much more sudden in onset. In short, we consider that the arrest of secretion caused by adrenaline is due to anoxia, as Langley supposed. When a condition of acute anoxia is suddenly produced by an intra-arterial injection of about 1 mg. KCN to an actively secreting gland, an arrest of secretion of very similar appearance is produced (Fig. 10B).

When the blood flow through the gland was observed, it was found (Fig. 11) that, in fact, the administration of anticholinesterase was accompanied by a considerably increased blood flow; ultimately a spontaneous secretion resulted, and the intra-arterial injection of adrenaline caused

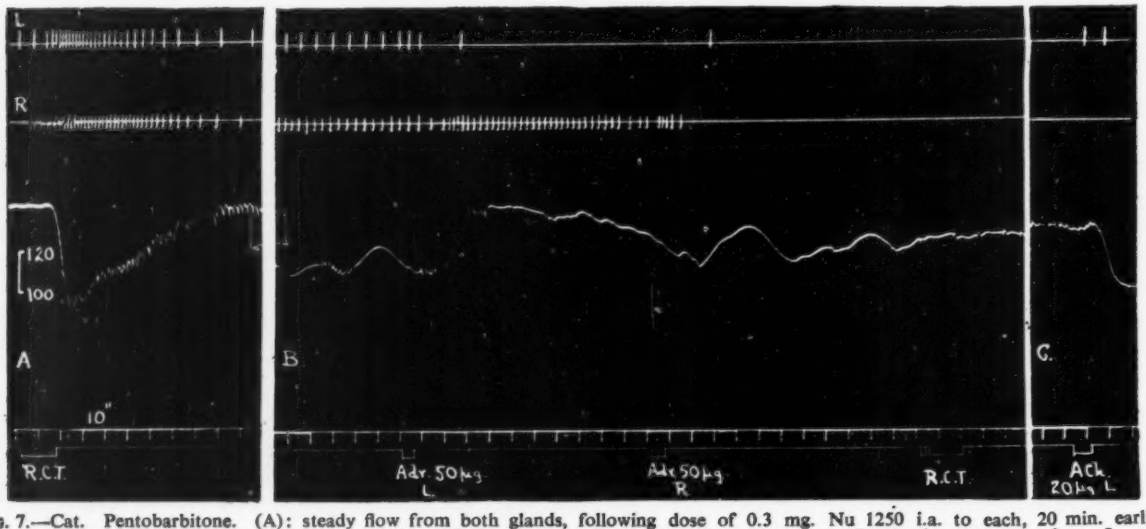

FIG. 7.—Cat. Pentobarbitone. (A): steady flow from both glands, following dose of 0.3 mg. Nu 1250 i.a. to each, 20 min. earlier was accelerated on both sides by stimulation of R chorda. (B): adrenaline, 50 μg. to L gland stops flow from L, and accelerates that on R; then 50 μg. to R gland checks its flow; the R chorda is then ineffective. (C): ACh 20 μg. to L produces small response 3 min. later; the initial response to 20 μg. ACh was 15–20 drops. (This cat had had L chorda divided 10 days before expt.)

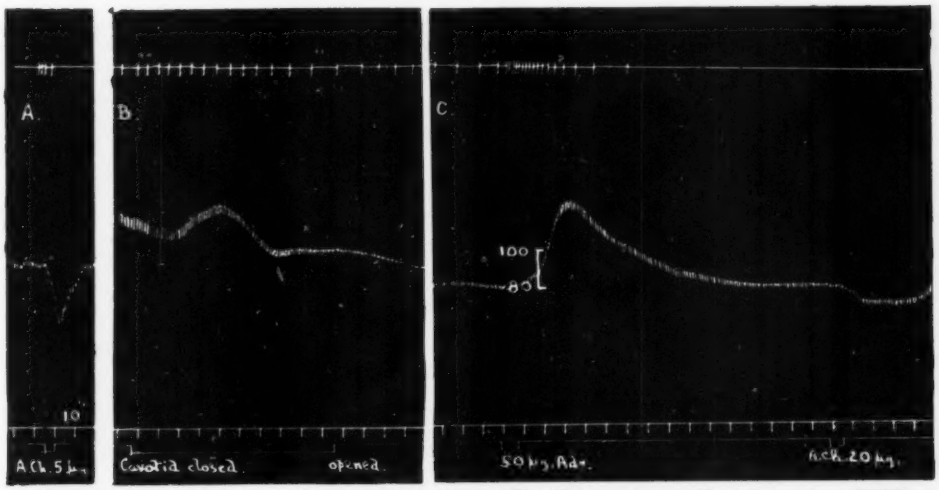

FIG. 8.—Cat. Pentobarbitone. (A): test dose of 5 μg. ACh. (B): spontaneous flow due to 1 mg. TEPP to gland 10 min. previously; effect of clipping of carotid artery. (C): effect of 50 μg. adrenaline in accelerating and then stopping spontaneous flow; during arrest 20 μg. ACh to gland gave no response. The arrest lasted 4 min.

both blood flow and secretion to be almost stopped. What we did not expect, however, was that even in the normal previously non-secreting gland intra-arterial adrenaline also caused considerable slowing or even prolonged arrest of the blood flow, and not, as commonly stated, a brief check followed by an increase. The explanation of this discrepancy was found when adrenaline was given intravenously; this caused a brief check followed by considerable increase in the blood flow through the gland. It would seem probable that the rise of general arterial pressure, as well as the production of products of metabolism, are together responsible for the increased blood flow, the vasoconstrictor action of the adrenaline being passively overcome by the raised arterial pressure.

One of the consequences of the ischaemia and anoxia of the gland which follow when adrenaline is given after the production of a spontaneous flow is that the gland temporarily fails to respond to chorda stimulation (Fig. 7)

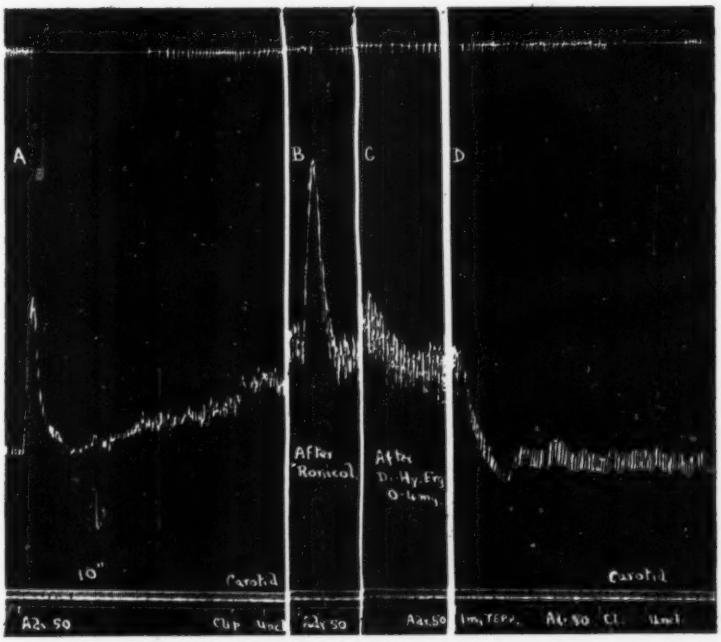

FIG. 9.—Same expt. as Fig. 8. Flow was increased by 1 mg. pilocarpine to gland. (A): adrenaline 50 μg. caused arrest; clipping off of carotid only slowing. (B): after 20 mg. "Ronicol," adrenaline only causes a stop of a minute or two. (C): after 0.4 mg. dihydroergotamine, adrenaline causes no slowing. (D): flow stimulated by 1 mg. TEPP is not arrested by 50 μg. adrenaline; closure of carotid artery now arrests the flow promptly.

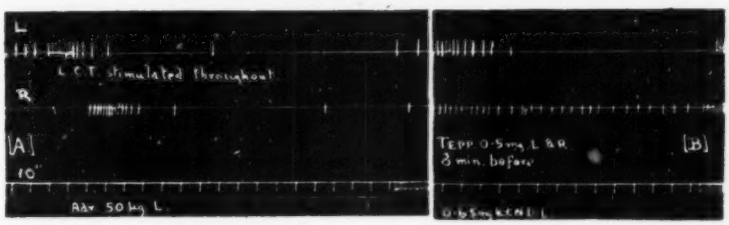

FIG. 10.—Cat. Pentobarbitone. (A): the L chorda was stimulated throughout. Intra-arterial injection of 50 μg. adrenaline to L gland first augmented and then stopped the flow; escaping adrenaline caused secretion of R gland. (B): flow started by TEPP, 0.5 mg. to each gland 8 min. before; injection of 0.65 mg. KCN into L gland stops flow abruptly.

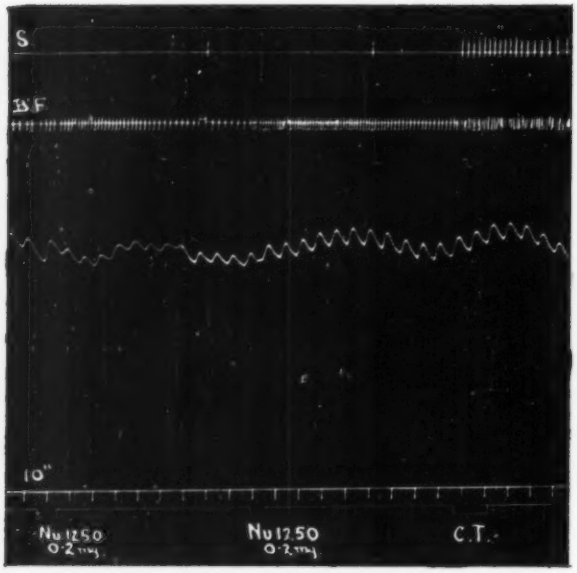

FIG. 11.—Cat. Pentobarbitone, heparin. S, drops of saliva from L gland. B.F., blood flow in drops from L gland. Two doses of Nu 1250 cause acceleration of blood flow, further accelerated by chorda stimulation; the flow of saliva was only slow.

and to ACh (Fig. 8); the latter evidently enters very slowly, as is shown by the slight effect which it produces on the arterial pressure, despite the dose of anticholinesterase (compare A and C, Fig. 8).

Graham and Stavraky (1953) also found that, whereas small doses of adrenaline intra-arterially cause mainly vasodilatation, large doses cause vasoconstriction, but with secretion also. But Stavraky (1942) finds, as we do, that when the gland is secreting spontaneously, under the influence of pilocarpine or eserine, small amounts of adrenaline increase, whereas large amounts diminish, the rate of secretion.

The Influence of Denervation

The left chorda tympani was sectioned or the left superior cervical ganglion removed under aseptic conditions, and the acute final experiments performed after the lapse of some days or weeks.

Section of the Chorda Tympani.—At the operation as great a length as possible of the chorda was removed, viz., from the point where the nerve left the chordo-lingual nerve up to or just beyond the point at which it ran alongside the duct, usually about 5–7 mm. In no instance was there a considerable loss of weight of the denervated gland at the time of experiment. It must be remembered that section of the chorda is only a decentralization, since, although some of the ganglion relay cells are scattered along its course, they are mainly aggregated near the hilum of the gland, and cannot be extirpated surgically. It was found by Chang and Gaddum (1933) that after section of the chorda the ACh content of the submaxillary gland fell considerably after 24 hr. and did not recover, but that sympathectomy had no effect on the ACh content. MacIntosh (1937) found no change in the cholinesterase activity of the gland after chorda section.

Incidentally, the relative sensitivity of the operated and intact glands to ACh and to adrenaline was tested. Opinions differ as to the effects of ACh after chorda section; some claim that the sensitivity of the gland is increased (Wills, 1941; Graham and Stavraky, 1951), others that it is diminished (Fleming and MacIntosh, 1935; Pierce and Gregersen, 1937).

Our experiments were inconclusive as regards the effect of chorda section on the sensitivity to ACh. Out of 11 cats the sensitivity to doses of ACh of the order of 1 to 10 μg. was increased in 3, unchanged in 4, and reduced in 4; in one dog it was reduced. The time after operation varied from 5 to 19 days, but within these limits did not seem to affect the results.

We concluded at the time that any change which was found after chorda section was due to some unknown incidental factors, and that there was no clear evidence that the sensitivity was necessarily altered by the operation in the sense that Cannon's law would demand. The important contribution of Graham and Stavraky (1953), which appeared while this paper was in course of preparation, does, however, seem to explain the discrepancies—including those found in our experiments—and will be referred to later. One peculiarity which we noticed was that, even when the total response of the two sides to ACh was the same, the rates of secretion differed, that on the denervated side always being more sluggish than that on the normal side (Figs. 12 and 14).

In testing the action of anticholinesterases some days or weeks after chorda section, we found that the gland on the denervated side was sometimes earlier and sometimes later in showing the characteristic effects of potentiation of ACh action and of the yielding of spontaneous secretion, but that the effects were complicated by the fact that it seemed to be more easily damaged or fatigued than the normal gland. This was especially noticeable when sarin was used, which was unfortunately the case in four of the experiments. This substance seemed to have a damaging effect on the denervated side, lowering all responses, and this led to the paradox of a reversal of the effects of

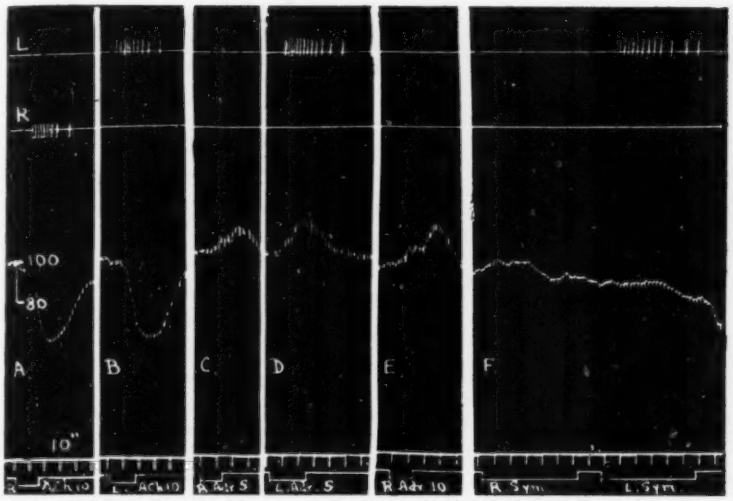

FIG. 12.—Cat. Pentobarbitone. Left chorda divided 7 days before. (A), (B): response of L and R glands to 10 μg. ACh. (C) and (D): responses of R and L sides to 5 μg. adrenaline. (E): response of R side to 10 μg. adrenaline. (F): responses to stimulation of R and L cervical sympathetic.

ACh on the two sides. Before the dose of sarin, the denervated side (L) was the more sensitive to both ACh and adrenaline; after the sarin, the response on the L side to both was greatly reduced, owing, we believe, to damage, whereas the response to both ACh and to adrenaline, following the usual course, was potentiated on the normal (R) side after the anticholinesterase.

There were no very striking differences in the action of TEPP or of Nu 1250 on the two sides. Small doses gave potentiation of the effects of ACh on both sides, and generally the threshold required on the denervated side was somewhat reduced, as compared with the other side. When larger doses were given, the spontaneous flow sometimes started sooner on the denervated side (Figs. 13 and 14), but was slower than that on the normal side; also, it tended to die out, as though from fatigue, leaving the normally innervated side the more affected.

In his studies on the pupil after decentralization or removal of the ciliary ganglion, Anderson found that pilocarpine was more, and eserine less, effective than on the normal side, eserine being in fact without action on the denervated pupil (Anderson, 1905b). It may be, therefore, that the varying results we got could be attributed to the removal of varying amounts of ganglia on the chorda.

In one experiment we tried the response of the two sides to pilocarpine, since Langley (1885) and Fleming and MacIntosh (1935) stated that it gave a smaller secretion on the denervated side, while Pierce and Gregersen (1937) and Emmelin and Muren (1951) found a greater one. Our results (Fig. 15) agreed with the latter finding, but the first rapid secretion from the denervated side was eventually outlasted by that from the normal, which developed more slowly.

The sensitivity of the chorda-denervated side to adrenaline, and to stimulation of the cervical sympathetic, was found to be clearly enhanced in most experiments (Fig. 12); where it was diminished, the response to ACh was also diminished, and it is perhaps worth mentioning that in 3 such cases out of 4 the cats were abnormally large and old; we agree with the findings of Fleming and MacIntosh (1935) and Emmelin that after chorda section the gland is more sensitive to adrenaline.

Sympathetic Denervation.—This was effected by surgical removal of the superior cervical ganglion; the acute part of the experiment was carried out a few weeks later. The denervated side, as expected, proved to be clearly more sensitive to adrenaline than the normal side; to ACh there was no difference. The action of the anticholinesterase appeared to differ in no way from that on the normally innervated gland.

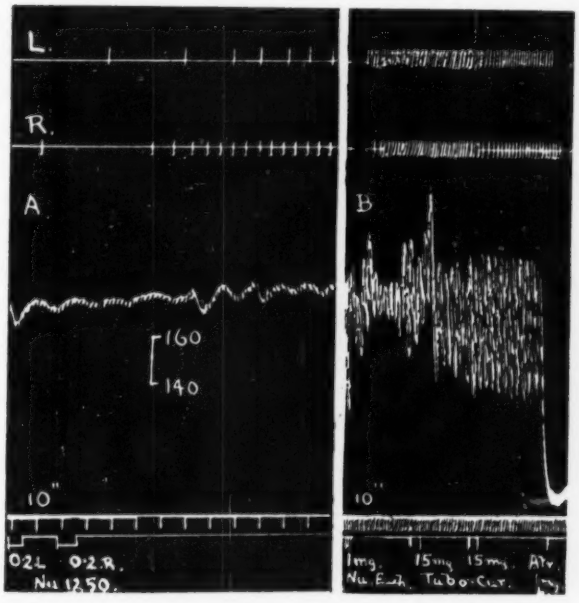

FIG. 13.—Cat. Pentobarbitone. L chorda cut 7 days previously. (A): Nu 1250 0.2 mg. to L and R glands. (B): 30 min. after A, flow has slackened; 1 mg. Nu 1250 (making a total of 1.4 mg. to each) given to each gland restarts it. Two doses of 15 mg. tubocurarine (making a total of 40 mg. to the two glands) fail to arrest flow; atropine 1 mg. arrests it instantly.

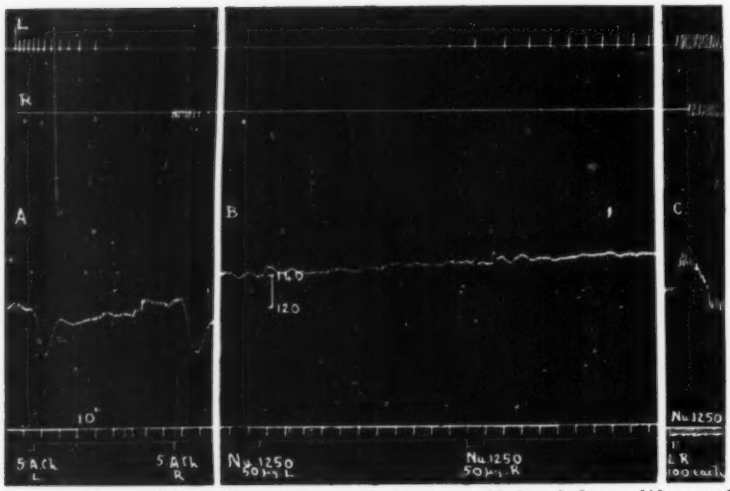

FIG. 14.—Cat. Allobarbitone-urethane. L chorda cut 12 days before. (A): equal but differently spaced responses to 5 μg. ACh on two sides. (B): Nu 1250 50 μg. first to L and then to R gland starts off flow from L, not from R. (C): Nu 1250 to L and R glands (100 μg. each) starts off flow first from L.

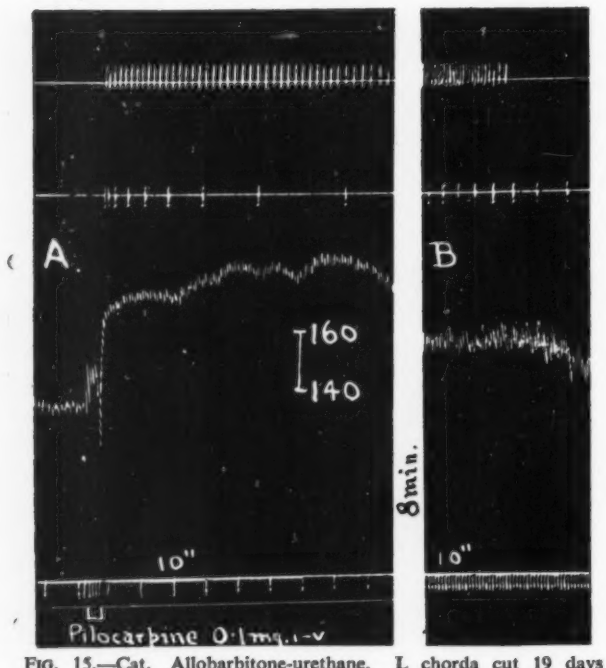

FIG. 15.—Cat. Allobarbitone-urethane. L chorda cut 19 days before. (A): pilocarpine 0.1 mg. i.v. starts off rapid flow from L gland, slow one from R gland. (B): 8 min. later, flow from L gland stops suddenly, that from R continues for some minutes.

DISCUSSION

All the anticholinesterases we have tried, when given in suitable doses, caused the potentiation of the effects of ACh in small amounts or of stimulation of the chorda tympani, and all of them when given in sufficiently large doses caused a spontaneous secretion of saliva. No evidence has been encountered which precluded the hypothesis that these effects were due to the action of these agents in inhibiting cholinesterase, except the general toxic effects of large doses, seen especially with sarin, and which may mean that some other enzyme systems are then made ineffective. When the drugs were given by close arterial injection the most pronounced effects tended to be confined to the sites of administration, despite some evidence of general dispersal of the anticholinesterase or of its decomposition products. The explanation of the potentiated action of small doses of ACh or of chorda stimulation seems obvious enough on the basis of cholinesterase inhibition. As regards the production of the spontaneous flow, and granted that the action is peripheral, there could be two explanations. The first would be that the anticholinesterases directly stimulate the secreting cells in addition to inhibiting cholinesterase; as a variant of this it could be supposed that they cause liberation of ACh as well as delaying its destruction.

The other, and more likely, explanation would be that there is normally, and independently of any arrival of nervous impulses, a constant liberation of ACh in the tissues of the gland, but that the rate of liberation is too slow to allow of the concentration, faced with the dual losses due to diffusion and to the action of cholinesterase, reaching a threshold value. The anticholinesterase, by checking one of these sources of loss, allows the concentration of ACh to mount up to a level controlled by diffusion alone, and adequate to cause a continuous secretion from the gland. There is evidence in the literature, to which reference has already been made, of such a liberation of ACh by the submaxillary gland, though it is not unanimous, and we have not fully confirmed it. In order to do so, and to exclude the possibility that the anticholinesterase might itself liberate ACh, it would be necessary to demonstrate the presence of ACh in venous blood or in saline perfusion effluent from the submaxillary gland without the addition of eserine or other anticholinesterase. But it is not improbable that a slow liberation of ACh is a universal phenomenon, with only quantitative differences from one tissue to another. We have, for instance, the example of the intestine, to which reference has already been made, and of the production of the miniature end-plate potentials in striated muscle by liberation of ACh (Fatt and Katz, 1952). In

the salivary glands of ruminant animals, moreover, there is a state of constant secretion, which could probably be attributed to such a liberation normally reaching values over the threshold. The constant state of secretion of the sublingual gland in the cat (Emmelin, 1953) probably does not fall into this category, since it is not stopped by atropine.

Unless the spontaneous flow is maximal, it can be accelerated by injection of small doses of ACh or by chorda stimulation, though larger doses of ACh or more prolonged or powerful stimulation of the chorda can produce "block"—a phenomenon which can also be demonstrated on the unpoisoned gland.

Small doses of atropine check the spontaneous flow at once, and thereafter the administration of even huge doses of anticholinesterase fails to restart it. This is understandable in view of what has been said. Atropine is regarded as raising a barrier to the action of ACh; when the barrier is adequate to keep back that concentration of ACh, which accumulates when all the cholinesterase has been inactivated, and if a state of equilibrium has been established between rate of ACh production and the rate of its dissemination by diffusion, any further dose of anticholinesterase, assuming it does not increase the rate of ACh production, could not overcome the atropine barrier. With pilocarpine or with ACh matters are different, since these probably act similarly and at similar sites; hence it is possible, in a series of administrations alternatively of pilocarpine and atropine, to start and stop the spontaneous secretion, as was shown by Langley (1880) and as we have verified.

Ganglion-blocking agents given by close arterial injection, or nicotine painted on the ganglia, do not arrest, though they may slow down, the spontaneous flow; the slowing is probably due to the considerable fall in arterial pressure which these agents cause, and not, we think, to interference with an excitatory state in the ganglia. The action causing spontaneous flow is therefore a peripheral one.

The spontaneous flow is accelerated by adrenaline or by sympathetic stimulation, and this would seem to be a phenomenon akin to the familiar accelerated or "augmented" secretion. If, as we suppose, the spontaneous flow is due to the ACh liberation having been rendered effective by inactivation of the cholinesterase, it would be reasonable to regard the gland as being virtually in a condition of parasympathetic excitation, and so to be liable to be highly sensitive to adrenaline.

The striking arrest of the spontaneous flow which is caused by the close arterial injection of large doses (e.g., 50 μg.) of adrenaline, and which has been noted in the literature without having been explained, may be attributed to the combination of raised glandular metabolism with a long-lasting ischaemia of the gland. It occurs also when the flow of saliva has been produced by chorda stimulation or by pilocarpine, and is not therefore peculiar to the state of anticholinesterase poisoning. Our experiments on the blood flow through the gland have shown that such large doses of adrenaline intra-arterially do cause prolonged ischaemia both in the normal and in the spontaneously secreting gland.

One of the main objects of our study was to find whether any correlation could be found between the type of cholinesterase inactivated and the production of such physiological effects as the spontaneous flow of saliva. Although the results were not so clear-cut as we hoped for, they did show that, for equal degrees of inactivation, that of the true cholinesterase was the more effective. In order to result in the production of a spontaneous flow it would appear that at least some 60% of the true cholinesterase must be put out of action, whereas the inactivation of some 80% of the pseudo-cholinesterase did not lead to a spontaneous flow, though it might produce some potentiation of the action of ACh or of chorda stimulation. In general agreement with these observations, Riker and Wescoe (1949) found that the maximal decrease of threshold of chorda stimulation occurred with DFP dosage when the total ChE content of the gland had been reduced to about 10% of its original value; and that at about the same level the spontaneous flow began. Reduction to 50% they found to have no physiological effects.

As regards the effects of chorda section, we were unable to demonstrate any regular alteration in the sensitivity of the gland to ACh, such as might resolve the existing discrepancy in the literature. One feature was invariable, viz., that the denervated gland always secreted more slowly than the normal one; but we can offer no explanation for this. It was found by MacIntosh (1937) that the cholinesterase content of the gland was unaltered after chorda section.

The work of Graham and Stavraky (1953), which appeared during the preparation of this paper, seems to have given the explanation. They have shown that doses of ACh above a certain level cause vasoconstriction in the gland, with reduced secretion. This is no doubt the same effect which

we have attributed to block from excess ACh. Moreover, they find that this effect is considerably enhanced in the denervated gland. It is not possible directly to compare the doses we used with those given by Graham and Stavraky. We did not confine the arterial supply to the gland alone as they did, so that to produce results similar to theirs our intra-arterial doses were much larger. Our doses ranged from 1 to 10 μg., or occasionally more, whereas theirs were from 10^{-4} to 10 μg. on the small-dose range—though from 50–1,000 μg. in the large dose-range. It is probable that, since the effect is enhanced by denervation, the doses we used were of an order that tended to slow down the effect on the denervated gland and not on the normal one. We believe that in accordance with the recent work of Graham and Stavraky it may be accepted that the gland is more sensitive to ACh after chorda section, provided that small doses are involved. This would explain our finding that, on the balance, the operated gland was found to be more sensitive to anticholinesterases. We also found it more sensitive to pilocarpine.

We have also confirmed the now well-established fact that after chorda section there is an increased sensitivity of the gland to adrenaline. This augmentation of sensitivity to a close relative of the sympathetic transmitter, after decentralization of the parasympathetic supply, is apparently not an isolated phenomenon. The converse of it, an increase of sensitivity to ACh after sympathetic denervation, was found by Graham and Stavraky (1953). It is also, in a way, paralleled by the phenomenon of "paradoxical pupil constriction" seen after section of the third nerve or removal of the ciliary ganglion when the animal is anaesthetized and partially asphyxiated, i.e., under conditions in which adrenaline is liberated (Anderson, 1905a). The effect of adrenaline on the denervated pupil is paradoxical inasmuch as it resembles the action of the parasympathetic, though differing from it, as does the potentiation of the effect of adrenaline on the (chorda-) decentralized salivary gland, in being unaffected by small doses of atropine.

The effects of anticholinesterases on sympathetically denervated or parasympathetically decentralized glands did not differ in any essential respect from their effects on normal glands. The slightly more prompt response on the side with chorda divided might have been due to small differences in blood flow, or to the state of subliminal excitation due to an actual or incipient paralytic secretion. MacIntosh (1937) found no change in the cholinesterase content of the gland as a result of chorda section; in any case, the paralytic secretion is not to be attributed to an increased release of ACh, since, as we have verified, it is not stopped by atropine, and moreover Chang and Gaddum (1933) found the ACh content of the gland to be reduced after chorda division.

The experiments, however, do show that the action of anticholinesterases on the salivary glands is almost wholly, if not entirely, due to a peripheral effect, exercised, it may be supposed, on the nerve endings of the chorda. These endings are inconspicuous, and the spatial relation of the cholinesterase sites and of the site of production or liberation of ACh to them is obscure; it is not even known with any certainty whether the endings are inside the secreting cells or not, though there is some evidence that they are to be found only in the mucous cells, the serous cells being supplied by the sympathetic (Babkin, 1950). Whatever their site and relations, however, it seems reasonable to believe that there is a constant liberation of ACh from some place near to or within the secreting cells, that the cholinesterase is near to that place, so that this slowly liberated ACh never reaches such a local concentration in the resulting gland cells as to cause them to secrete. Stimulation of the chorda, by liberating ACh at a greatly accelerated rate, or anticholinesterases, by retarding the rate of its destruction, enable enough of it to reach the cells to cause them to secrete; the potentiation of the action of ACh or chorda stimulation by anticholinesterases is self-explanatory.

The phenomena of the augmented secretion have been a puzzle to physiologists for the past half century or more, and enter here into our problem. Explanations have usually amounted to little more than a re-statement of the facts of experiment. It certainly seems odd that the activity of one type of nerve should augment that of the other, especially since the functions of the sympathetic and parasympathetic supply to many organs are apparently opposed, and seem to have little in common, their chemical transmitters being different. Looked at from the standpoint of modern theories of excitation, however, they have probably one thing in common—that the state of excitation is always the result of a depolarization of a polarized membrane. Even if we accept the view that the sympathetic and parasympathetic fibres are supplied to cells of different types, we might admit that some membrane in the mechanism of the acinus supplied by the sympathetic fibre was depolarized by adrenaline, while that of the other type was depolarized by ACh. These cells are in close histological proximity to each

other, they may be bounded on one side by a common membrane—the basement membrane ; and it does not seem beyond the bounds of possibility that in such adjacent cells, especially if they are of slow rates of accommodation, the depolarization of the bounding membrane of a cell of one type might induce a facilitated state in a contiguous membrane bounding an adjoining cell. Until some means of putting this explanation to the test of experiment can be devised, it must remain a speculation.

Summary

1. When an anticholinesterase is administered by close arterial injection to the gland much of it is retained by the gland tissues. The effect is, first, to potentiate doses of ACh or a stimulus to the chorda tympani, then, as further doses are given, to cause a spontaneous secretion of saliva.

2. The less readily reversible anticholinesterases, when given by close arterial injection, stay firmly and for long periods in the gland first dosed, while the more reversible ones may pass on and even affect the opposite gland.

3. The spontaneous flow of saliva is caused by a purely peripheral action, and is unaffected by section, and but slightly affected by degeneration, of the nerve supply. It is slowed, but not stopped, by ganglion-blocking agents.

4. The spontaneous flow of saliva produced by an anticholinesterase is hastened by ACh or by chorda stimulation, except when the flow is already maximal. It is also hastened by adrenaline ; this phenomenon is a variant of Langley's phenomenon of " augmented secretion." Large doses of adrenaline, however, when injected intra-arterially, stop the spontaneous flow for long periods ; this effect is due to local vasoconstriction and anoxia, enhanced by raised metabolism.

5. In order to produce spontaneous flow, a large fraction of the true cholinesterase must be inhibited ; inhibition of a larger amount of the pseudo-enzyme, if not accompanied by considerable inhibition of the true enzyme, does not produce a spontaneous flow, though potentiation of the action of ACh may be shown.

6. The production of the spontaneous flow is explained as being due to the revealing, by inhibition of cholinesterase, of a constant production of ACh, normally insufficient to cause secretion, by the gland.

7. Block phenomena attributable to the presence of excess ACh can be demonstrated in the salivary gland ; the explanation of this is similar to that for neuromuscular block. No block is produced by anticholinesterases unless ACh is also given or produced by chorda stimulation.

8. The phenomenon of augmented secretion may perhaps be explained on the modern theories of excitation.

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THE ACTIONS OF ANTIMONIALS ON GLYCOLYTIC ENZYMES OF *SCHISTOSOMA MANSONI*

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It is generally assumed that the chemotherapeutic action of organic antimonials is brought about by mechanisms similar to those of arsenicals—that is, by interaction with, and inactivation of, sulphydryl enzymes (Findlay, 1950). Both arsenicals and antimonials have chemotherapeutic activity against the same parasitic protozoa, such as certain species of trypanosomes and leishmania. On the other hand, antimonials are effective in schistosomiasis whereas arsenicals are not. In *Schistosoma mansoni*, glycolysis proceeds at an exceedingly rapid rate, and this metabolic process is more essential for the survival of the worms than is respiration. It has been found previously that glycolysis in intact worms is inhibited by organic antimonials (Bueding, 1950). In the present paper the effects of antimonials on glycolysis of worm homogenates, and on some of their glycolytic enzymes, are reported.

METHODS

The adult worms were obtained as in previous studies (Mansour and Bueding, 1953). The parasites were homogenized in cold potassium glycylglycine buffer (0.01M, *p*H 7.5) in an all-glass homogenizer; 1 ml. of buffer was used for each 100 pairs of worms. The final molar concentrations of the constituents in the medium, optimal for glycolysis of the homogenates, were as follows: potassium glycylglycine buffer, 5×10^{-2} (*p*H 7.5); glucose, 8×10^{-3}; adenosine triphosphate (ATP), 5×10^{-3}; diphosphopyridine nucleotide (DPN), 7×10^{-5}; nicotinamide, 3×10^{-3}; magnesium chloride, 8×10^{-3}. Schistosome homogenates were shaken in this medium at 38° C. for 1 hr. The mixtures were deproteinized by the addition of barium hydroxide and of zinc sulphate (Somogyi, 1945a). For the determination of preformed lactic acid control samples were deproteinized before incubation. Lactic acid was determined according to Barker and Summerson (1941). The same procedure was used for measuring the rate of glycolysis in the presence of two other substrates, fructose-6-phosphate (F-6-P) and fructose-1,6-diphosphate (HDP). Potassium arsenate (final concn. 1×10^{-3}M) was present in the medium when HDP was used as the substrate. Glucokinase, fructokinase and mannokinase activities were measured by incubating the enzyme preparations of the worms with the hexose and with ATP in the presence of magnesium chloride, for a period of 10 min. at 38° C. The final molar concn. of the constituents optimal for this assay were: glucose or mannose, 1.5×10^{-3}; fructose, 1.6×10^{-2}; ATP, 7×10^{-3}; $MgCl_2$, 1×10^{-2}; potassium glycylglycine buffer (*p*H 7.5), 5×10^{-2}. The hexose was added to the experimental samples before incubation and to the control samples immediately after incubation. Protein and phosphate esters were precipitated with barium hydroxide and zinc sulphate (Somogyi, 1945a). The concentration of the non-phosphorylated hexose was determined in the filtrate (Somogyi, 1945b). Hexose utilization was calculated from the difference between the free hexose concn. of the control (reaction mixture incubated without the hexose) and that of the experimental sample. Purification of glucokinase and of phosphohexose isomerase was carried out according to Bueding and MacKinnon (unpublished methods). Phosphohexose isomerase activity was measured by incubating (38° C., 15 min.) the enzyme preparation of the worms in media containing glucose-6-phosphate (molar concn. 1.25×10^{-2}) and potassium glycylglycine buffer (*p*H 7.5; molar concn. 5×10^{-2}). Proteins were precipitated with one volume of trichloroacetic acid (15% w/v). The concn. of the fructose ester formed was determined by the method of Roe (1934) as modified by Higashi and Peters (1950). Phosphofructokinase activity was determined by incubating the enzyme in a medium containing potassium glycylglycine buffer (*p*H 7.5; 5×10^{-2}M), magnesium chloride (8×10^{-3}M), ATP (8×10^{-3}M) and F-6-P (1×10^{-2}M) for 20 min. at 38° C. Proteins were precipitated in the experimental tubes after, and in the control tubes before, incubation, by adding 9 volumes of trichloroacetatic acid (5% w/v). Enzymatic activity was determined by the rate of disappearance of acid-labile phosphate resulting from the transfer of phosphate from ATP to F-6-P: after heating the protein-free filtrate for 7 min. in 1N-HCl in a boiling water-bath, phosphate was determined according to Gomori (1942). Phosphofructokinase from rat brain was prepared by the method of Muntz (1953).

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Results

Effect of Antimonials on Lactic Acid Production from Glucose.—Two trivalent antimonials, stibophen and antimony potassium tartrate, had a marked inhibitory effect when glucose was used as the substrate (Table I). On the basis of these observations an attempt was made to determine which enzyme or enzymes were responsible for the reduction of glycolysis produced by the antimonials.

TABLE I

EFFECT OF ANTIMONIALS ON LACTIC ACID PRODU CTION BY EXTRACTS OF *SCHISTOSOMA MANSONI*

Antimonial	Molar Concn.	μMoles Lactic Acid/mg. Protein/1 Hr.		% Inhib.
		Control	With Antimonial	
Antimony potassium tartrate	1×10^{-3}	2·75	0·15	95
	1×10^{-4}	2·5	0·7	72
	4×10^{-4}	2·5	2·1	15
Stibophen	1×10^{-3}	2·75	0	100
	5×10^{-4}	2·75	0·0375	98
	8×10^{-4}	2·5	0·65	74

Effect on Hexokinases.—The first enzyme tested was glucokinase, which catalyses the initial step in the utilization of glucose, namely, its phosphorylation to glucose-6-phosphate by ATP. Both the purified and the crude enzyme preparations were relatively insensitive to high concentrations of antimonials (Table II). At a concentration of

TABLE II

EFFECT OF ANTIMONIALS ON GLUCOKINASE ACTIVITY OF *SCHISTOSOMA MANSONI*

Activities are expressed in μmoles of glucose phosphorylated per mg. protein in 10 min. (37° C.)

Enzyme Preparation	Molar Concn.	Control	Antimony Potassium Tartrate		Stibophen	
			Activity	% Inhib.	Activity	% Inhib.
Purified enzyme	1×10^{-2}	2·2	1·55	28	1·06	51
	3×10^{-3}	3·2	2·94	8	3·2	0
	1×10^{-3}	1·2	1·6	0	1·85	0
Schistosome homogenate	$1·6\times10^{-2}$	0·63	0·76	0	0·43	31
	3×10^{-3}	0·75	0·75	0	0·65	13
	1×10^{-3}	0·75	0·62	17	0·86	0

1×10^{-3}M both stibophen and antimony potassium tartrate produced complete inhibition of glycolysis but did not affect hexokinase activity. Other hexokinases, fructokinase and mannokinase, which are present in this parasite (Bueding and MacKinnon, 1953), were also insensitive to high concentrations of the antimonials. Therefore, inhibition of hexokinase cannot account for the inhibition of glycolysis of schistosomes by antimonials. On the other hand, the purified enzyme preparations, as well as homogenates of the worms, were markedly inhibited by low concentrations of oxophenarsine (mapharsen) and of other sulphydryl inhibitors, such as *p*-chloromercuric benzoate. For example, at a concentration of 5×10^{-4}M, oxophenarsine inhibited the activity of purified glucokinase to an extent of 83%.

Phosphohexose Isomerase.—This enzyme catalyses the second step in glycolysis, namely, the reversible conversion of glucose-6-phosphate to fructose-6-phosphate. Neither stibophen nor antimony potassium tartrate, at concentrations below 1×10^{-2}M, inhibited enzymatic activity significantly (Table III). The above observations

TABLE III

EFFECT OF ANTIMONIALS ON THE ACTIVITY OF PURIFIED PHOSPHOHEXOSE ISOMERASE OF *SCHISTOSOMA MANSONI*

Activities are expressed as μmoles of fructose-6-phosphate produced per mg. protein in 15 min. (37° C.)

Molar Concn. of Antimonial	Control μmole F-6-P	Antimony Potassium Tartrate		Stibophen	
		μmole F-6-P	% Inhib.	μmole F-6-P	% Inhib.
1×10^{-2}	520	390	25	415	20
5×10^{-3}	470	425	10	450	3
1×10^{-3}	520	435	16	480	8
5×10^{-4}	470	470	0	490	0

excluded the first two reactions of glycolysis as the site of the inhibitory action of these drugs.

Effect of Antimonials on Lactic Acid Production from F-6-P and HDP.—In order to localize the effect of the antimonials, their action on lactic acid production from HDP and from F-6-P was measured. When HDP was used lactic acid production was not affected even by high concentra-

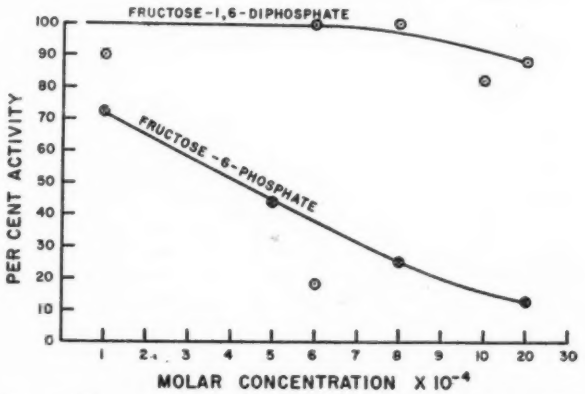

FIG. 1.—Effect of antimony potassium tartrate on lactic acid production from F-6-P and from HDP by schistosome extracts. Abscissa: molar concn. of antimonial. Ordinate: activity (% of control).

tions of antimony potassium tartrate (Fig. 1). On the other hand, with F-6-P as the substrate the activity of the extract was markedly decreased at molar concentrations above 5×10^{-4}. Similar results were obtained with stibophen (Fig. 2). These data indicated that the inhibition of glycolysis by antimonials is brought about by blocking the

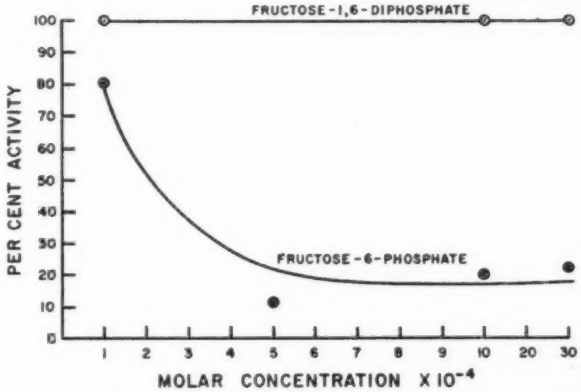

FIG. 2.—Effect of stibophen on lactic acid production from F-6-P and HDP by schistosome extracts. Abscissa: molar concn. of stibophen. Ordinate: activity (% of control).

formation of HDP from F-6-P. The latter reaction is brought about by the action of phosphofructokinase, which catalyses the phosphorylation of F-6-P to HDP by ATP.

Effect of Antimonials on Phosphofructokinase.—The data summarized in Table IV demonstrate that the activity of the phosphofructokinase of schistosomes was inhibited markedly by low concentrations of trivalent antimonials. On the other hand, the

TABLE IV

INHIBITION OF PHOSPHOFRUCTOKINASE ACTIVITY OF *SCHISTOSOMA MANSONI* AND OF RAT BRAIN BY ANTIMONIALS

Molar Concn. of Antimonial	Antimony Potassium Tartrate		Stibophen	
	Worm Enzyme % Inhib.	Brain Enzyme % Inhib.	Worm Enzyme % Inhib.	Brain Enzyme % Inhib.
1×10^{-2}		100		0
4×10^{-3}	100	54	100	
1×10^{-3}	100	32	100	0
5×10^{-4}	100	4	100	
3×10^{-4}	100	0	85	
1×10^{-4}	70	0	44	0
5×10^{-5}	56	0	19	
3×10^{-5}	32	0	0	
1×10^{-5}	2	0	0	0

enzyme, which catalyses the same reaction in the host is much less sensitive to the same antimonial compounds (Table IV). Concentrations of tartar emetic 80 times higher than those necessary to inhibit the worm enzyme were required to produce the same degree of inhibition of the brain enzyme. With stibophen the difference was more striking still because even the highest concentration used had no effect on the mammalian enzyme. Oxophenarsine (5×10^{-4}M) completely inhibited the activity of phosphofructokinase of schistosomes. As with antimonials, the enzyme of rat brain was much less sensitive to oxophenarsine (Table V).

TABLE V

EFFECT OF OXOPHENARSINE ON PHOSPHOFRUCTOKINASE ACTIVITIES OF *SCHISTOSOMA MANSONI* AND OF RAT BRAIN

Molar Concn.	Worm Enzyme % Inhib.	Brain Enzyme % Inhib.
2×10^{-3}	—	88
5×10^{-4}	100	0
1×10^{-4}	73	0
4×10^{-5}	36	—
1×10^{-5}	17	—

In contrast to the two trivalent antimonials, high concentrations (4×10^{-3}M) of a pentavalent antimonial, sodium stibogluconate (Schmidt, 1948), had no effect on the activity of the worm enzyme.

DISCUSSION

It is evident that in schistosomes low concentrations of trivalent antimonials inhibit one reaction of glycolysis, the phosphorylation of F-6-P to HDP. By contrast, a pentavalent antimonial did not affect the activity of phosphofructokinase of the worms. This is in agreement with the finding of Lee and Chung (1935) that pentavalent antimonials do not exert a direct lethal action on schistosomes *in vitro*. It would appear that, as with arsenicals, pentavalent antimonials are reduced by the tissues of the host before chemotherapeutic activity is obtained. According to Chen (1948) trivalent antimonials as well as trivalent arsenicals have an inhibitory effect on three glycolytic enzymes of *Trypanosoma equiperdum*—hexokinase, adenosine triphosphatase and 3-phosphoglyceraldehyde dehydrogenase. This multiple action on trypanosome enzymes might explain the effectiveness of these compounds as chemotherapeutic agents in African trypanosomiasis. Whereas the activities of the schistosome hexokinases are inhibited by low concentrations of oxophenarsine, these enzymes are not affected by trivalent antimonials, even at high concentrations. Therefore, it appears that inhibition of enzymatic activity by these two groups of compounds is not brought about by the same mechanism. It should be noted also that enzymes catalysing the same reactions in different species are not affected by the same inhibitors. In contrast to

the schistosome hexokinases, hexokinase of *T. equiperdum* is inactivated by low concentrations of antimonials (Chen, 1948). Furthermore, the phosphofructokinase of the host is much less sensitive to trivalent antimonials and to trivalent arsenicals than is the phosphofructokinase of schistosomes. These observations, as well as those reported previously (Mansour and Bueding, 1953, and Mansour, Bueding and Stavitsky, 1954), indicate that enzymes which have the same catalytic function in the parasite and in the host are not necessarily identical. Although the selective action of trivalent antimonials on phosphofructokinase of *S. mansoni* explains the inhibition of the rate of glycolysis of the worms, it remains to be determined whether this effect accounts for the mechanism of the chemotherapeutic action of antimonials in schistosomiasis.

Summary

1. The action of trivalent antimonials and of oxophenarsine on the glycolytic enzymes of *Schistosoma mansoni* was investigated.

2. Stibophen and antimony potassium tartrate had little effect on the parasite's hexokinase, which is very sensitive to oxophenarsine.

3. Trivalent antimonials did not inhibit the activity of the parasite's phosphohexose isomerase, or the production of lactic acid from hexose diphosphate by schistosome extracts, but they had a marked inhibitory action on the phosphofructokinase of the worms.

4. The sensitivity of phosphofructokinase to trivalent antimonials accounts for the inhibitory effect of these compounds on the rate of glycolysis of *S. mansoni*.

5. Phosphofructokinase of rat brain was much less sensitive to trivalent antimonials and to oxophenarsine.

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THE EFFECTS OF LEAD, MERCURY, AND GOLD ON THE POTASSIUM TURNOVER OF RABBIT BLOOD CELLS

BY

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Some heavy metals cause a loss of potassium from rabbit erythrocytes (Henriques and Ørskov, 1936; Davson and Danielli, 1938). There is normally a slow exchange of K between rabbit erythrocytes and the surrounding fluid, and this exchange can be demonstrated with radioactive tracer (Mullins, Fenn, Noonan and Haege, 1941). The potassium concentration in the cells is about twenty to twenty-five times that in plasma. This ratio is much greater than that observed with chloride or hydrogen ions; the difference in concentration does not therefore depend on a Donnan equilibrium, and involves the forced inward movement of ions by metabolic activity as well as movement in both directions by diffusion through the more or less permeable cell walls. These points have been fully discussed by Harris and Maizels (1952). Net loss of K from cells is due either to an increased permeability of the cell walls, so that the work done by the cells is insufficient to offset the exaggerated outward leak of K; or to a decrease in the work done by the cells, so that the normal efflux of K is no longer counteracted by adequate pumping of K inwards; or both. With the first change the rate of entry of tracer into cells immersed in a medium containing ^{42}K would be unaltered or accelerated, and with the second the rate of entry would be diminished. In order to examine these possibilities we have examined the movements of K between rabbit erythrocytes and their environment in the presence and absence of the chlorides of lead, mercury and gold, with the results described below.

METHODS

Treatment of Cells.—In order to avoid changes in cells associated with washing and centrifuging (Davson and Danielli, 1938), the inward passage of tracer was followed when fresh rabbit blood was added to a slightly modified Krebs' solution containing ^{42}K and, when appropriate, small quantities of $PbCl_2$, $HgCl_2$, or $AuCl_3$. The solution was composed of NaCl 0.120M, $NaHCO_3$ 0.025M, $CaCl_2$ 0.0025M, $MgSO_4$ 0.0012M, KCl 0.0042M, and dextrose 0.2% (w/v); O_2 + 5% CO_2 was bubbled through it for 20 min. before use to give a *p*H of 7.4. The KCl was made up from irradiated K_2CO_3 which contained ^{42}K (half life 12.4 hr.): the activated K_2CO_3 was converted to KCl by dissolving weighed amounts in N-HCl and diluting appropriately. Blood was obtained by venepuncture or cardiac puncture from apparently healthy rabbits. 5 ml. portions were placed in tared 30 ml. stoppered centrifuge tubes, weighed and left for 15–30 min. in a water bath at 37° C. or in the refrigerator at 7°. 20 ml. portions of salt solution were kept in the same way. To begin each experiment, the Krebs' solutions were poured into the samples of blood. The tubes were shaken gently to mix their contents, weighed rapidly, and put on a rotary mixer turning at 7 rev./min. in an incubator at 37° or in a refrigerator at 7°.

Sampling.—From time to time 2 ml. samples were removed from the mixtures and were centrifuged for 18 min. at 3,000 rev./min. and 15 cm. radius (1,500 *g.*). The tubes used had a bore of 3 mm. for the bottom 40 mm. and 8 mm. for the upper 45 mm., with a tapering junction 10 mm. long. The cells did not completely fill the narrow segment of the tube, and their volume was determined from the length of the cell column measured directly against a millimetre scale. The tubes were calibrated with known volumes of fluid added from a micrometer syringe. The supernatant fluid was separated without disturbing the cell column and the part of the tube above the cells was twice washed with 5% (w/v) dextrose. The washings were added to the supernatant fluid, which was then made up with water or dilute NaCl to a volume (25–100 ml.) suitable for estimating total K and radioactivity. The quantity of NaCl used in diluting was such that the final Na concentration was approximately 0.002M. 1 ml. of 0.05% (w/v) saponin was added to the cells. These were mixed and transferred with washings to 100 ml. volumetric flasks, and made up for estimations like the supernatant fluids. An allowance of 5% of the cell volume was made for trapped supernatant fluid, and the estimated cell concentrations were so corrected. The actual volume trapped may have varied considerably (Jackson and Nutt, 1951), but this average correction is consistent with previous observations under similar conditions (Ponder, 1948; Leeson and Reeve, 1951), and was

supported by experiments with Krebs' solution containing ^{24}NaCl from which the initial uptake of ^{24}Na by the cells is slow.

Estimations of Potassium and Radioactivity.—Potassium was estimated on a flame photometer, similar to that described by Holiday and Preedy (1953) as modified by Neil (1953), in the range 0.5 – 4 × 10^{-4}M by comparison with appropriate standard solutions of KCl in 0.002M-NaCl. The accuracy of the estimations was ± 0.04 × 10^{-4}M (S.E. of duplicate determinations). Radioactivity of the solution was determined from duplicate counts with an M.R.C. type M.6 liquid counter and a Dynatron type 1009B scaling unit, with suitable corrections for background activity and decay and resolution time. Usually at least 10,000 impulses were counted, giving a theoretical standard error of ± 1%, and the agreement between duplicate counts was of this order.

Calculation of the Minimum Quantities of an Ion Species Entering and Leaving Cells in a Given Period.—For unit quantity of cells, let the quantity of ions of a particular species moving through the cell walls be Q_i inwards and Q_o outwards in a given period. Let the amount of ion in the cells at the beginning and end of the period be b_1 and b_2 moles and the radioactivity y_1, y_2. Let the specific activity of the medium be A_p and that of the cells A_c at the beginning of the period. Then the quantity of ions of the particular species moving inwards cannot be less than the increment in cell radioactivity divided by the original specific activity of the medium; and the quantity moving outwards cannot be less than the difference between the amount of ion in the cells at the beginning and end of the period (with due regard to sign). Thus,

$$Q_i \geqslant \frac{y_2 - y_1}{A_p} \quad (1)$$

and

$$Q_o \geqslant b_1 - b_2 \quad (2)$$

The estimated inward movement is too small because it does not allow for any loss of radioactive ions from the cells during the period, and it does not allow for the decrease in the specific activity of the medium due to the efflux of inactive ions from the cells. Some allowance can be made for the loss of radioactive ions by using expression (2) and the initial specific activity of the cells, giving

$$Q_i \geqslant \frac{y_2 - y_1}{A_p} + (b_1 - b_2).\frac{A_c}{A_p} \quad (3)$$

Also, the outward movement must be large enough to balance the inward movement as well as the net change in cell ionic content. So

$$Q_o \geqslant b_1 - b_2 + \frac{y_2 - y_1}{A_p} + (b_1 - b_2).\frac{A_c}{A_p} \quad (4)$$

Progressive approximations can be obtained by continuing to adjust the inward and outward estimates alternately, and the series obtained converges more or less rapidly according to the magnitude of the ratio A_c/A_p. The result obtained is still an underestimate because it does not take into account changes in specific activity of the medium and cells during the period, and in practice it has not been worth proceeding further. The estimated minimum movements given in Tables II and III are obtained from the expressions (3) and (4).

Results

Untreated Cells.—Typical results are shown in Table I and in Figs. 1 and 2. As we have been concerned with the changes in poisoned cells which usually began to haemolyse after a few hours at 37°, we have studied normal cells at this temperature only for comparably short periods, in which time the amount of potassium which exchanged was rather small for a satisfactory estimate of the rate to be made. In the first four to six hours it makes very little difference whether the uptake of ^{42}K by the cells is regarded as linear or as the beginning of an exponential approach to equili-

TABLE I

THE EFFECT OF LEAD CHLORIDE ON THE EXCHANGE OF POTASSIUM BY RABBIT BLOOD CELLS AT 37° C.

Time: Min.	Cell Vol.: % Suspension	K⁺ μ.equiv. in Cells of 1 ml. Suspension	K⁺ μ.equiv. in 1 ml. Cells	Specific Activity Counts/ μ.equiv.
A. *Normal Cells*				
0	—	—	101	0·0
X	6·3	6·4	102	1·5
30	6·2	6·3	102	4·4
90	5·8	6·3	109	9·6
120	5·9	6·4	109	11·6
With complete exchange	—	—	—	112·0
B. *Cells in Medium containing* $0{\cdot}8 \times 10^{-5}$M-$PbCl_2$				
0	—	—	101	0·0
X	6·3	6·3	101	2·4
30	5·7	5·2	92	10·8
60	5·5	5·0	92	11·7
90	5·5	4·9	90	16·2
120	5·6	5·1	91	17·8
With complete exchange	—	—	—	112·0
C. *Cells in Medium containing* $1{\cdot}6 \times 10^{-5}$M-$PbCl_2$				
0	—	—	101	0·0
X	6·2	6·2	101	7·3
30	4·9	3·8	78	44·6
60	4·8	2·9	61	51·0
90	4·9	2·8	57	51·9
120	4·9	2·6	53	57·0
With complete exchange	—	—	—	112·0

At beginning of experiment, all media contained 6·0 μ.equiv./ml. K^+ at 229 counts/μ.equiv.

At end of experiment, medium A contained 5·8 μ.equiv./ml. K^+ at 226 counts/μ.equiv.

At end of experiment, medium B contained 7·0 μ.equiv./ml. K^+ at 184 counts/μ.equiv.

At end of experiment, medium C contained 9·8 μ.equiv./ml. K^+ at 127 counts/μ.equiv.

(X: samples taken as soon as possible after mixing; centrifugation begun within 7 min. of mixing.)

brium with the suspending medium (Sheppard and Martin, 1950). Moreover, a small net gain of K, similar to that described by Danowski (1941), often occurred during this period in the untreated cells (though not in the experiment shown in Table I), and much K was usually lost from the poisoned cells, so that movements in opposite directions through the cell membranes were unequal and calculations of the "exchange rate" were not usually applicable to our conditions. Instead, minimum values for the entry and exit of K in the cells have been calculated and are shown in Table II. It will be observed that the rates of movement, at least inward, were greater at first than later. Similar observations have been made before (Sheppard and Martin, 1950; Raker, Taylor, Weller and Hastings, 1950), and their interpretation is uncertain. At 7° the rates of movement were of the order of one-tenth of those observed at 37° (Table III); there was a slow loss of K from the cells, barely detectable in the first 24 hr. and varying from 4 to 15% of the total cell K in 72 hr.

Cells Treated with Lead Chloride.—In all the experiments, concentrations of heavy metals are recorded in terms of the quantity of metal per unit volume of entire suspension. In fact erythrocytes take up lead rapidly from saline suspensions (Mortensen and Kellogg, 1944), and the final concentration of lead per unit volume of cells may have been up to twenty times greater than the concentrations which are given for the entire suspension. In some experiments, not otherwise reported here, in which a larger proportion of blood to saline was used, the same total concentrations of lead had much less effect, as would be expected if the lead was distributed over a greater number of cells. As the cell suspensions discussed here all contained about the same proportion of cells, and as the amount of lead fixed is uncertain, the total concentration is the most convenient one to use. Comparisons between the concentration of different metals producing similar effects are not significant, as the amounts of mercury or gold fixed by the cells are probably not even approximately equal to the amounts of lead which are fixed.

Lead chloride in sufficient concentrations caused a loss of K from erythrocytes (Table I and Figs. 1 and 2). The curves relating the quantity of K in the cells to time were approximately exponential in shape and are conveniently described by the new level which the cell K approached and by the half-time of the approach, as has been done in

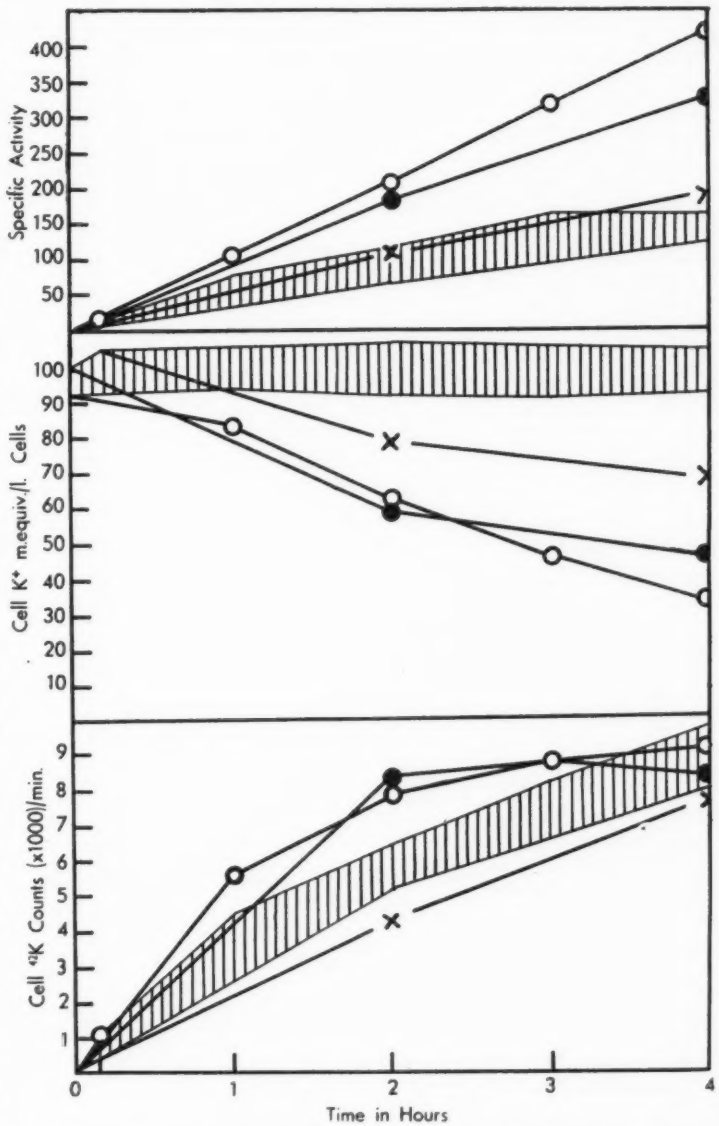

FIG. 1.—The effect of low concentrations of $HgCl_2$, $PbCl_2$, $AuCl_3$ on rabbit blood cells at 37°. Top ordinate: Specific activity, counts/min./m.equiv. cell potassium. Middle ordinate: Potassium, m.equiv./l. of cells. Bottom ordinate: Radioactive potassium, counts/min. Abscissae: Time, hours. ×——× $HgCl_2$ $3{\cdot}2\times10^{-5}$M. ●– –● $PbCl_2$ $0{\cdot}8\times10^{-5}$M. ○——○ $AuCl_3$ $2{\cdot}7\times10^{-5}$M. Range of control observations shaded.

Table II. The limiting level S_∞ has been obtained either graphically or by the expression

$$S_\infty = \frac{S_0S_3 - S_1S_2}{S_0 + S_3 - S_1 - S_2}$$

where S_0, S_1, S_2 and S_3 are successive estimates of the quantity of K in the cells either at constant time intervals or such that the interval between S_0 and S_1 is the same as that between S_2 and S_3 (Johnson, 1952). The half-time has been obtained by fitting straight lines by eye to values of $\ln\frac{S-S_\infty}{S_0-S_\infty}$ plotted against time (Fig. 3). Except when small errors in the original measurements had a large effect on the magnitude of the transformed values, most of the results were quite well fitted in this way.

The K concentration approached in the cells appears to depend somewhat on the concentration of Pb. Small changes were observed in 0.8×10^{-5}M-$PbCl_2$, rather variable ones in 1.6×10^{-5}M and in this or any higher concentrations up to 6.4×10^{-5}M the largest losses of K, about 75% of the total contained in the cells, were observed. With still higher concentrations of Pb, haemolysis of some of the cells occurred within an hour, and the changes were not studied in detail. The rate at which the cell K fell was not clearly related to the concentration of Pb.

The cells also lost water in the first half hour after exposure to lead, but not subsequently. As a result, changes in the K concentration in the cells follow a somewhat different course from changes in the total quantity of K, and, on the whole, the changes in concentration were less well fitted by an exponential relationship. A few observations on the cell Na showed that Na was gained as K was lost; but the molecules of Na gained were fewer than those of K lost, and the total (Na+K) in the cells was at least approximately proportional to the cell volume.

These changes were accompanied by a surprising and large increase in the rate of entry of ^{42}K to the cells, so that the total radioactivity of the cells was commonly ten or twenty times that of the untreated controls half an hour after the beginning of the experiment. After about this time, long before the net efflux of potassium was complete, the radioactivity diminished again, and there was usually little further increase in the specific activity of the cells from this time onwards. The changes in the early part of the experiment were rapid, and in view of the difficulty of determining the exact time of separation of cells and supernatant fluid, the course of the uptake and loss of tracer was

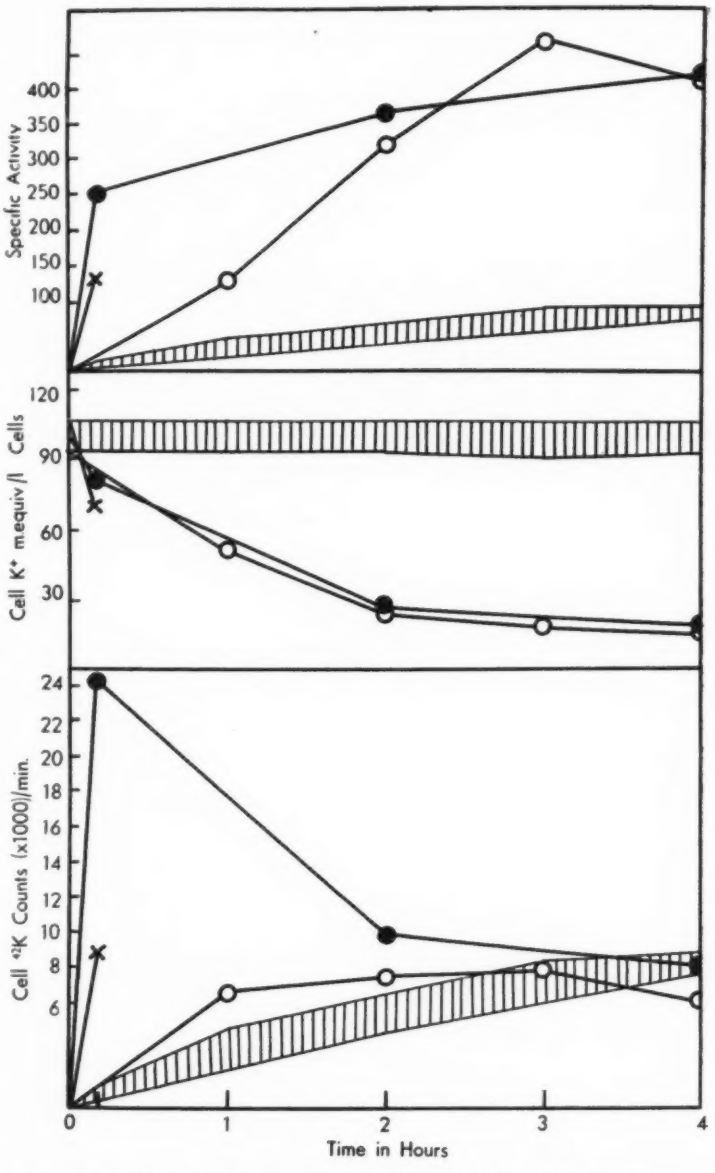

FIG. 2.—The effect of high concentrations of $HgCl_2$, $PbCl_2$, $AuCl_3$ on rabbit blood cells at 37°. Ordinates and abscissae as in Fig. 1. ×——× $HgCl_2$ $6\cdot4\times10^{-6}$M. ●——● $PbCl_2$ $1\cdot6\times10^{-5}$M. ○——○ $AuCl_3$ $5\cdot4\times10^{-5}$M. Range of control observations shaded.

TABLE II

MOVEMENT OF POTASSIUM INTO AND OUT OF RABBIT BLOOD CELLS AT 37° C.
(All values as % of original K^+ concentration)

Condition	Net Change in Cell K^+		Minimum Inward Movement/hr.				Minimum Outward Movement/hr.		
	Equilibrium Level	Half-time of Approach to Equilibrium (min.)	Mode of Calculation	0–½ hr.	0–2 hr.	2–4 hr.	0–½ hr.	0–2 hr.	2–4 hr.
Normal ..	99·4–103·6	—	A	2·6– 4·8	1·3–4·1	1·3–1·7			
			B	—	1·3–2·7	1·1–1·6	0–7·4	0–2·9	0–2·1
$PbCl_2$ $0·8\times10^{-5}$M	*ca.* 87·4	*ca.* 18	B	6·6–19·0	3·3–6·0	—	37·8–44·0	15·3	5·7
$1·6\times10^{-5}$M	18·9–47·3	31–38	B	19·8–33·4	6·8	0·6	101·2–124·6	34·8–42·3	2·9
$3·2\times10^{-5}$M	27·3	16	B	26·8	9·6*	—	151·8	35·7	1·7
$6·4\times10^{-5}$M	27·9	17	B	25·8	11·9*	—	142·4	61·9	—
$HgCl_2$ $3·2\times10^{-5}$M	*ca.* 100·0	—	A or B	—	2·0	2·0	—	2·0	0
$6·4\times10^{-5}$M	59·0	88	B	—	2·2	2·0	—	12·1	7·4
$AuCl_3$ $0·5\times10^{-5}$M	*ca.* 100·0	—	A or B	—	3·1	1·6	—	0–1·7	1·7–2·8
$2·7\times10^{-5}$M	< 36·0	—	B	—	5·6	2·0–3·0	—	39·6	3·5
$5·4\times10^{-5}$M	15·1–16·8	—	B	—	2·5–3·5	1·8	—	10·2–39·2	6·5–20·5

A = Calculated by the method of Sheppard and Martin (1950). B = Calculated by linear approximation. * = 0–90 min. figure.

not followed in detail, and the time and the magnitude of the peak of the curve showing the uptake of ^{42}K by the cells have not been determined accurately. The linear interpolations in Figs. 1 and 2 are a convenient way of connecting related points rather than an attempt to represent the true course of the reaction. The mechanism of these changes is discussed later, but it seems worth remarking that this greatly increased flow of K into the cells occurred against a substantial concentration gradient, and decreased rapidly and almost completely before the cell K finished falling. It is also notable that the specific activity showed little subsequent change, although it was not identical in cells and plasma; usually about half to three-quarters of the total possible exchange had taken place at this time. With the higher concentrations of lead, haemolysis began to occur after one or two hours, and with the lower concentrations after about four to twenty hours, and observations were not continued when this began to happen.

Minimum rates of entry and exit of K are shown in Table II: their calculation is subject to much larger errors than the calculation for normal cells; but all the errors tend to make the figures too small, and the actual rates may well be two or more times the estimated minima. Nevertheless, the estimated rates of entry in the first two hours vary from two to six times those normally occurring, whereas the minimal rates of exit are up to sixteen times the normal, and are therefore undoubtedly very much increased.

At 7° similar changes occurred more slowly, so that the cell K did not reach its minimum until 24–48 hr. or longer, and the peak in the cell radioactivity was reached after two or three hours. One difference was very striking; this was the

TABLE III

MOVEMENT OF POTASSIUM INTO AND OUT OF RABBIT BLOOD CELLS AT 7° C.
(All values as % of original K^+ concentration)

Condition	Net Change in Cell K^+		Minimum Inward Movement/hr.				Minimum Outward Movement/hr.		
	Equilibrium Level	Half-time of Approach to Equilibrium (min.)	Mode of Calculation	0–2 hr.	0–24 hr.	24–48 hr.	0–½ hr.	0–24 hr.	24–48 hr.
Normal ..	92·0–100·0	—	B	0·9	0·10–0·17	0·06–0·11	0	0·11–0·20	0·18–0·35
$PbCl_2$ $0·8\times10^{-5}$M	28·3	1,560	B	6·9	0·60	0·13	14·6	2·7	1·20
$1·6\times10^{-5}$M	16·9–18·8	448–620	B	22·9*	0·20–1·00	0·09–0·10	16·3–32·4	3·0–4·3	0·13–0·30
$HgCl_2$ $1·6\times10^{-5}$M	> 80	—	B	—	0·13	0·05	—	0·20	0·30
$6·4\times10^{-5}$M	> 80	—	B	0·20	0·13	0·08	0·20	0·35–3·6	0·50
$12·8\times10^{-5}$M	Not calculable		B	3·4	—	—	43·6	—	—
$AuCl_3$ $5·4\times10^{-5}$M	4·1	1,860	B	—	0·20	0·33	0	1·23	1·65
$10·8\times10^{-5}$M	Not calculable		B	0·6	1·10	—	2·0–2·9	3·4–4·5	0·05

B = Calculated by linear approximation. * = 0–60 min. figure.

absence of haemolysis, in concentrations up to 3.2×10^{-5}M, even when the cells were maintained on the rotor for up to 72 hr.; at 37° the same concentration of lead was sufficient to cause gross haemolysis within four to eight hours.

Cells Treated with Mercuric Chloride.—Superficially, Hg had similar effects to Pb, but there were several differences of detail. Haemolysis appeared to depend more on the concentration of mercury than on the temperature; at either 7° or 37° lysis occurred within two hours after 12.8×10^{-5}M, and between 6 and 24 hr. after 6.4×10^{-5}M. Any lysis that was observed was always extensive, whereas after lead the appearance of haemoglobin in the supernatant fluid was often very gradual and not necessarily the harbinger of prompt generalized destruction of the cells. Potassium loss from Hg-poisoned cells was either rapid and followed quickly by haemolysis, or much slower than from Pb-poisoned cells and towards a level not less than half the original cell concentration. There was no appreciable change in cell volume, and the entry of tracer was accelerated only slightly (when the net changes were not extensive) or considerably when the cells were about to haemolyse. No striking flow of tracer back from the cells to the medium occurred, but the exchange rate of the poisoned cells probably fell after a time to levels below those of the untreated controls. At 7° the changes were similar though slower, and, as at 37° the range between inactive and haemolytic concentrations was not large, detailed studies were not made.

Cells Treated with Gold Chloride.—Again there was a broad resemblance to the effects of Pb, and differences of detail. In most respects the effects of Au lay between those of Pb and of Hg. Haemolysis occurred less quickly and less extensively than after Hg; at 37° concentrations of Au sufficient to cause appreciable loss of K began to cause lysis after about 6 hr. The K losses themselves were usually greater in the second and third hours than in the first hour at 37°, or about the sixth to tenth hours at 7°. Correspondingly, the exponential transformation of the results did not give a straight line, and the nearest approach to such a line is shown in Fig. 3. Accelerated uptake of ^{42}K was greater than after Hg (taking as comparable concentrations those which produced an approximately equal loss of cell K) but less than after Pb, and the time of the maximum uptake corresponded to the time of the maximum loss of K from the cells. Slight swelling of the cells occurred rather than shrinkage, and it remains to be seen how this relates to changes in the cell Na content.

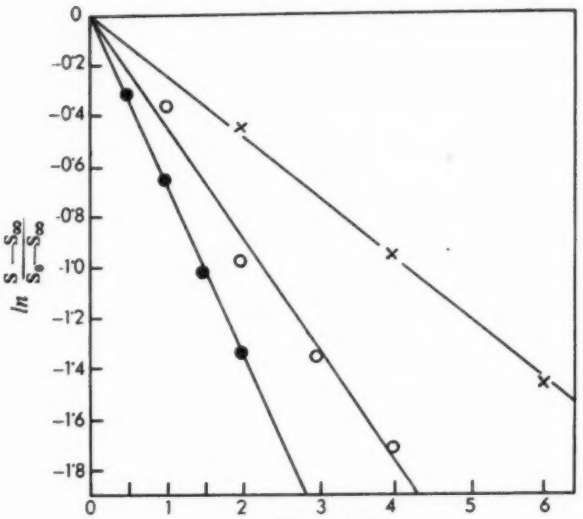

FIG. 3.—The rate of loss of potassium from poisoned rabbit blood cells at 37°. Ordinate: $\ln \frac{S - S_\infty}{S_0 - S_\infty}$ (see text). Abscissa: Time, hours. ×——× $HgCl_2$ $3{\cdot}2 \times 10^{-5}$M. ●——● $PbCl_2$ $1{\cdot}6 \times 10^{-5}$M. ○——○ $AuCl_3$ $5{\cdot}4 \times 10^{-5}$M.

DISCUSSION

The most striking finding in these experiments was that the loss of potassium from poisoned cells was generally, and particularly after lead, accompanied by an accelerated entry of potassium into the cells. If the metal affected the cell potassium simply by increasing the permeability of the cells to the ion, the influx as well as the efflux would be accelerated. The absolute acceleration of the influx would be much smaller because of the much lower external concentration of potassium. The increments which we have observed appear to be too large to be accounted for in this way; also, the rate of inflow is much greater in the first half hour or so after poisoning than it is later, so a simple increase in permeability is not sufficient to account for the findings.

If the cells are regarded as consisting of a membrane which offers a fairly high but not complete resistance to the passage of potassium, and a pump which injects potassium (or ejects sodium, or both) against the concentration gradient, it is necessary to postulate also some homeostatic mechanism which controls the pump, since at 37° the cell potassium and sodium concentrations normally remain fairly constant even when the environmental concentrations are altered considerably (Raker *et al.*, 1950; Solomon, 1952; Streeten and Solomon, 1954). On these assumptions lead may be regarded mainly as damaging the cell

membrane, so that its resistance to the passage of potassium is greatly reduced. As soon as potassium leaks out of the cells, the activity of the pump is increased and the rate of entry of potassium is raised five- to ten-fold, though not sufficiently to make up for all that is leaking out. Later, possibly because there are insufficient metabolites to maintain this high rate of working for a long time, the pump decreases in activity and the cell potassium concentration settles down at a new level; possibly also at this stage the initial increase in the permeability of the cell membrane is followed by a decrease as it becomes further disorganized. With mercury and gold there is less injury to the cell membrane and more interference with the pump, either directly or by obstructing the metabolism which supplies the necessary energy, and so the cells lose potassium both because they are more leaky and because the pump fails to respond adequately to the decline in the cell potassium concentration.

This hypothesis may be correct, but it is perhaps not very useful in the present state of knowledge about permeability of membranes and about homeostatic pumps at the cellular level. Also, the rates of movement of potassium are evidently changing too rapidly and in too complicated a fashion for the present experiments in themselves to throw much light on the details of either process.

Such a pump-and-permeability hypothesis also presupposes that the cells are behaving uniformly and that their internal ionic concentration may be treated as constant throughout the population. This appears to be true of normal human erythrocytes (Raker *et al.*, 1950; Sheppard and Martin, 1950), but it seems quite likely that some cells will be more affected than others by poisons: this is certainly so after a time, when some cells haemolyse while others do not, and, *a priori*, it is probably better to assume that the early changes in the cells are not uniformly distributed over the whole population. In this case, measurements of the average rate at which the population of cells are admitting or extruding potassium have a very questionable significance, and, to take an extreme position, some of the present results can be explained if a proportion of the cells become so permeable to potassium and sodium that they equilibrate completely with the external medium during the experiments, while other cells remain entirely or almost entirely unaffected. (A somewhat similar argument has been advanced by Ponder (1951) in connexion with the potassium loss from human erythrocytes suspended in an isotonic K-free medium.) The extreme case of an all-or-none response is insufficient to account for the observed efflux of ^{42}K from lead-poisoned cells, though it may be applicable in mercury poisoning. But the examination of intermediate situations is complicated and does not appear profitable at present.

That all the observed changes under the influence of heavy metals show much the same dependence on a change of temperature from 37° to 7° suggests that they are acting on a single process rather than partly on permeability and partly on a pump; it is unlikely that these two processes are equally sensitive to temperature changes, or that the extent to which they are upset by poisoning is dependent on temperature just sufficiently to offset their initial difference. The delay in the onset of changes after exposure to gold also requires explanation; it may indicate that some metabolic process is inhibited for some time before its effects become apparent, or it may simply indicate that a longer period is necessary for the gold itself to react with its receptors.

Numerous other substances, probably including all haemolytic agents, cause loss of potassium and gain of sodium in erythrocytes (Davson and Danielli, 1938; Ponder, 1948; Maizels, 1951), but we have failed to find observations from which estimates of the actual rates of movement of ions into and out of the cells can be made. The present observations show that estimates of permeability based simply on net gains or losses of ion species (e.g. Davson and Reiner, 1942) can give more or less identical values with greatly differing rates of movement. Accurate treatment of the situation when the rates are changing rapidly is difficult, but is evidently necessary for any pharmacological examination of the control of cell electrolytes to be useful.

Summary

1. Changes have been observed in the K content and in the entry of ^{42}K into rabbit blood cells poisoned with lead chloride, mercuric chloride and gold chloride at 37° and 7°.

2. All three metals caused loss of K from the cells, and later (except lead chloride at 7°) haemolysis.

3. Lead chloride greatly accelerated the entry of ^{42}K into the cells, at the same time as net losses of K were occurring. These changes occurred both at 37° and 7°, but were followed by haemolysis only at 37°.

4. Mercuric chloride mainly caused loss of K from the cells, but with large losses and incipient haemolysis the ^{42}K entry was accelerated.

5. Gold chloride acted less rapidly than the other two substances, and then produced changes intermediate between their effects.

6. The interpretation of these changes is discussed.

We are indebted to Dr. E. R. Cole for his collaboration in the early stages of this work, and to Dr. A. G. Ogston for helpful discussions and for reading the manuscript. Part of the expenses of the work were defrayed by grants (to M. W.) from the Medical Research Council and the University of London Central Research Fund, and we thank these bodies for their support.

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CHOLINE 2 : 6-XYLYL ETHER BROMIDE; AN ACTIVE QUATERNARY LOCAL ANAESTHETIC

BY

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(RECEIVED JULY 20, 1954)

When examining nuclear substituted choline phenyl ethers for nicotine-like stimulant activity we found that intravenous injection of one of these, choline 2: 6-xylyl ether bromide (TM.10), produced the typical brief rise in the cat's arterial blood pressure which is characteristic of these compounds. Subsequent doses of the same compound, however, failed to produce any further response—even after a lapse of several hours. This rapid and prolonged tachyphylaxis appears to result from a powerful local anaesthetic action of the drug.

METHODS

Cats were used in all the experiments. They were given 1 mg./kg. of atropine sulphate intraperitoneally, after which anaesthesia was induced with ether and maintained by chloralose (100 mg./kg. i.v.). Arterial blood pressure was recorded from a carotid artery by a mercury manometer, and a venous cannula was inserted in the femoral vein. Other experimental procedures are indicated in the description of particular experiments.

RESULTS

The extent of the tachyphylaxis to TM.10 is shown in Fig. 1. This also shows that TM.10 has some slight adrenolytic activity but that the magnitude and duration of this cannot account for the failure of the second dose of TM.10 to produce a pressor effect.

Analogous effects on the nictitating membrane of the cat are shown in Fig. 2. In this experiment the cervical sympathetic nerve was cut on one side and on the other the superior cervical ganglion was removed. On the side with the intact ganglion the response to standard intravenous doses of adrenaline remained fairly constant, whereas the response to doses of TM.10 diminished rapidly. On the side with the ganglion removed, doses of TM.10 regularly produced relaxation, seen also to some extent on the other side following the stimulant effect; it is possible that this is due to a transient adrenolytic action against normally circulating adrenaline.

Further confirmation of the prolonged effect of TM.10 is given by Fig. 3, which shows the contractions of the nictitating membrane on intermittent electrical stimulation of the cervical sympathetic nerve. A single dose of TM.10 abolished all response within a few minutes, and this effect persisted for more than three hours.

From these results it seemed unlikely that the effects of TM.10 could be ascribed to adrenolytic, sympatholytic or ganglion-blocking actions. It therefore seemed possible that the compound might act by suppressing conduction in postganglionic sympathetic fibres.

This hypothesis was tested in an experiment the results of which are shown in Fig. 4. Contractions

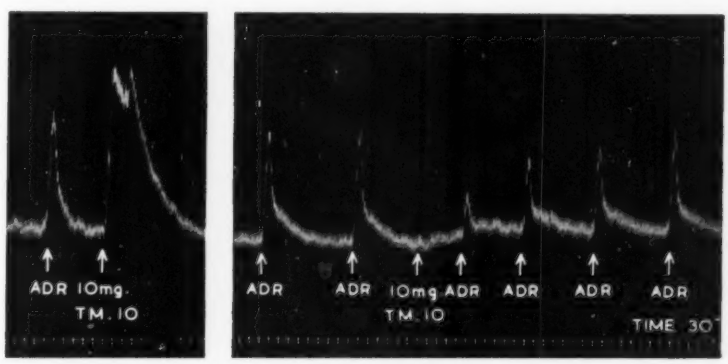


FIG. 1.—Carotid blood pressure of atropinized spinal cat showing tachyphylactic and adrenolytic actions of TM.10 administered intravenously. Adr = adrenaline 3 μg. i.v. Time interval between the two tracings is approximately 1 hr.

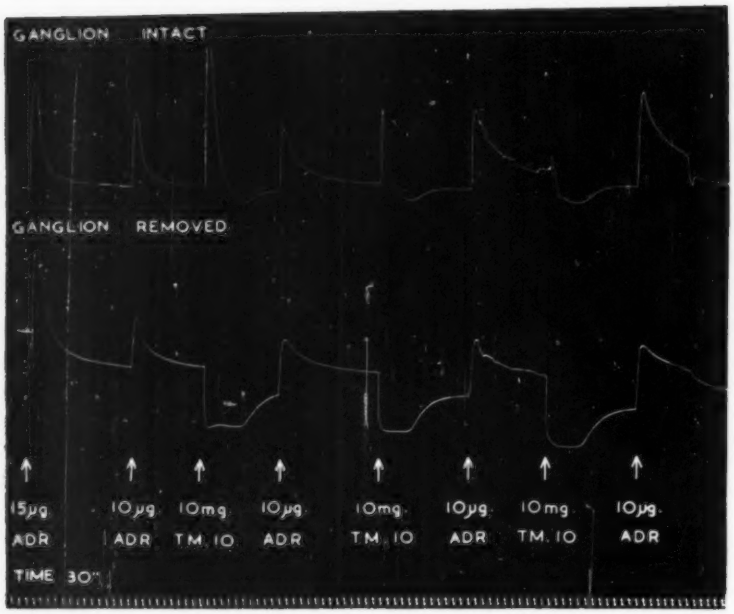


FIG. 2.—Cat, atropinized, chloralose. Responses of nictitating membranes to adrenaline and TM.10. Upper record from side with superior cervical ganglion intact. Lower record from side with ganglion removed at beginning of experiment.

of the nictitating membranes are recorded in response to intermittent electrical stimulation applied on one side to the postganglionic nerve trunk, and on the other to the cervical sympathetic nerve. The cervical sympathetic nerve was cut on both sides. After confirming the correct placing of the electrodes by an intravenous dose of tetraethylammonium iodide, TM.10 was given by the same route. A total dose of 20 mg. of TM.10 completely abolished responses in both membranes. (The residual small irregularities in the trace are due to unavoidable general motor stimulation from the postganglionic electrodes.) This, in conjunction with the other experimental data, made the hypothesis tenable, and, since postganglionic sympathetic nerve fibres resemble sensory fibres in being lightly myelinated, it seemed possible that TM.10 would show local anaesthetic actions.

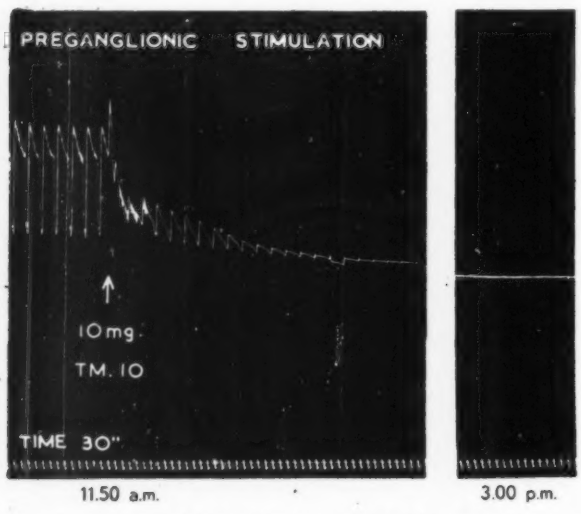


FIG. 3.—Cat, atropinized, chloralose. Nictitating membrane contractions. Periodic preganglionic stimulation (50/sec.) applied for 45 sec. in each min. At the arrow 10 mg. TM.10 injected i.v.

When tested by the cavy intracutaneous weal technique of Bülbring and Wajda (1945), TM.10 had a local anaesthetic activity approximately equal to that of cocaine hydrochloride. There were, however, very marked differences in the durations of the effect; the action of TM.10 lasted 2–10 times as long as that of cocaine, the larger differences being given by the higher concentrations. This difference in the duration of the effects is shown in Fig. 5, where the degree of anaesthesia is plotted, on a linear scale, against time on a logarithmic scale; at 0.3% concentration for both drugs it is evident that the effect of TM.10 lasts 5–6 times as long as does that of cocaine hydrochloride and that there is a detectable delay in the onset of action of the former. In preliminary experiments using a 2% solution of TM.10 the anaesthesia lasted approximately 48 hours.

Following these observations we demonstrated that a sufficiently large intravenous dose of procaine hydrochloride produced the same effects on the response of the nictitating membrane to electrical stimulation, but, as might be expected, these were of shorter duration. This is clearly shown in Fig. 6, in which the experimental conditions were the same as in Fig. 4.

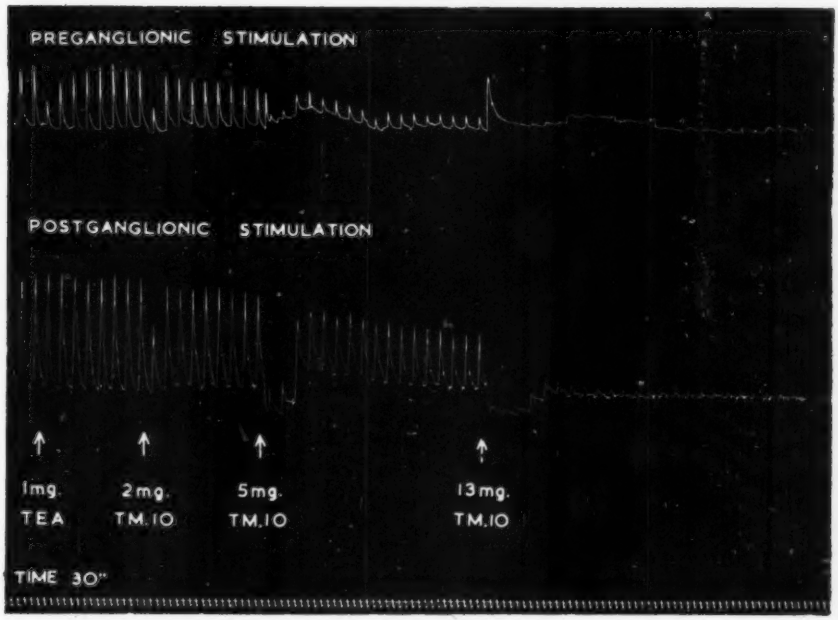


FIG. 4.—Cat, atropinized, chloralose. Contractions of nictitating membranes evoked by periodic stimulation (70 sec.) applied for 2 sec. in each min. to the preganglionic nerve (upper record) and to the postganglionic nerve (lower record). The arrows indicate injection of 1 mg. tetraethylammonium iodide, 2 mg. TM.10, 5 mg. TM.10, and 13 mg. TM.10 respectively.

Pronounced local anaesthetic activity in quaternary ammonium compounds has recently been described by Nádor, Herr, Pataky and Borsy (1953), but, in the compounds they studied, the onset of action was slow, whereas with TM.10 it is rapid.

We subsequently investigated a number of compounds related to TM.10; some data concerning these are presented in Table I. Local anaesthetic activity appears to depend on the presence of at least one suitable group in the ortho position to the side chain and that, given this condition, both quaternary and tertiary compounds are active. Topical activity is shown only by the tertiary compounds, which is in accord with the general conclusion that quaternary cations are poorly absorbed by mucous membranes.

Compound TM.10 has been tested in man by intracutaneous injection and the local anaesthetic properties have been confirmed.

In conclusion it is interesting to note that the more active compounds described show considerable steric similarities to lignocaine hydrochloride (" Xylocaine ").

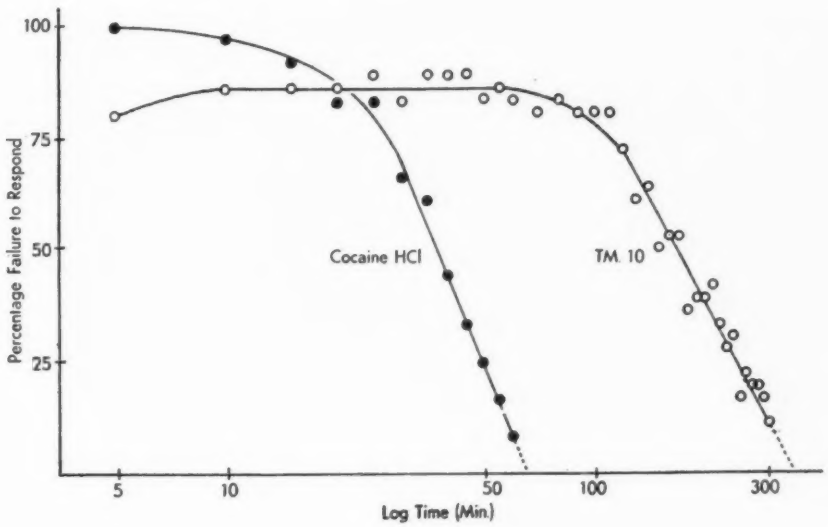


FIG. 5.—Graph showing the relative durations of local anaesthesia produced by intracutaneous injections of 0.3% cocaine hydrochloride and 0.3% TM.10. Ordinate, % failure of the guinea-pig to respond to mechanical stimuli. Abscissa, time (min.) on logarithmic scale.

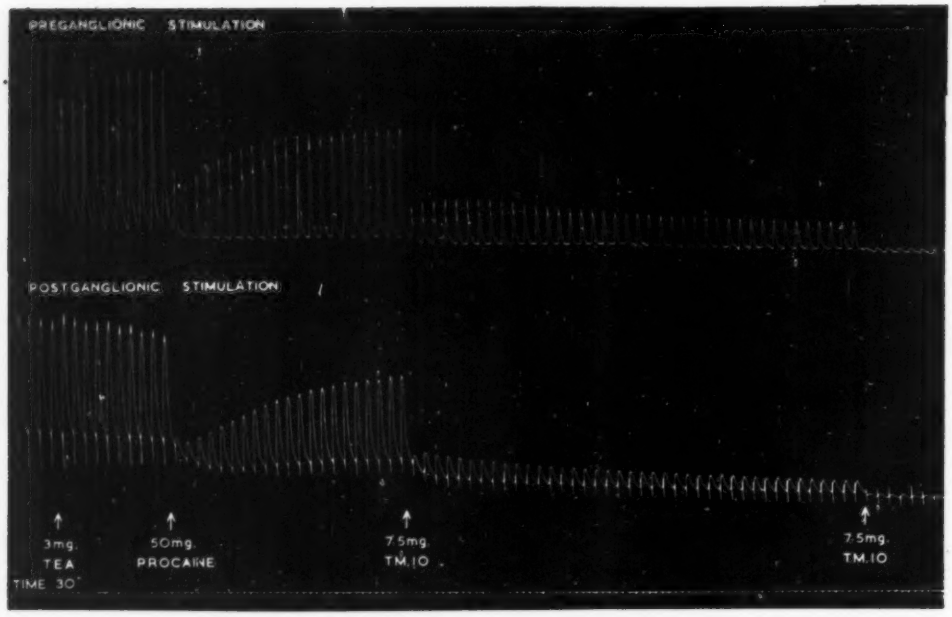
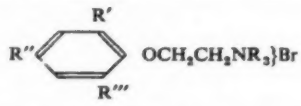
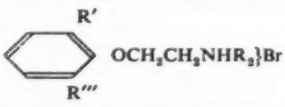


FIG. 6.—Cat, atropinized, chloralose. Contractions of the nictitating membranes in response to periodic stimulation (70/sec.) applied for 15 sec. in each min. The arrows indicate intravenous injections of 3 mg. tetraethylammonium iodide, 50 mg. procaine hydrochloride, 7.5 mg. TM.10, and 7.5 mg. TM.10 respectively.

TABLE I

LOCAL ANAESTHETIC PROPERTIES OF COMPOUNDS RELATED TO TM.10

Code Number	R	R′	R‴	R″	Anaesthetic Activity by Intracutaneous Injection (Cocaine HCl = 1)		Other Comments
					Activity	Duration	
Quaternary ammonium compounds							
TM.10	CH_3	CH_3	CH_3	H	1	2–10	No topical activity on rabbit cornea. LD50 (i.p. to mice) 95 mg./kg.
TM.1	CH_3	H	H	H	0	0	
TE.10	C_2H_5	CH_3	CH_3	H	1	1–5	No topical activity on rabbit cornea
TM.18	CH_3	CH_3	H	H	—	—	Intracutaneous dose of 1 mg. killed 11/12 cavies; one survivor showed anaesthesia. LD50 (i.p. to mice) 18 mg./kg.*
TM.17	CH_3	CH_3	CH_3	CH_3	1	2–10	
Tertiary amines							
TTM.10	CH_3	CH_3	CH_3	—	1	1	Shows topical activity on rabbit cornea approximately equal to cocaine
TTE.10	C_2H_5	CH_3	CH_3	—	1	1	Shows topical activity on rabbit cornea approximately equal to cocaine. LD50 (i.p. to mice) 95 mg./kg.

* Dr. K. A. Exley (private communication) has subsequently shown that the toxic effect of TM.18 results from a neuromuscular paralysing activity, approximately one-fifth that of (+)-tubocurarine chloride on a weight basis, and that the difference in toxicity by the intracutaneous and intraperitoneal routes is owing to slow absorption by the latter.

Chemical Section

Melting points and boiling points are uncorrected.

1. β-Aryloxyethyl bromides were prepared by methods similar to those previously described (Hey, 1952). The following are recorded:

(a) *β(2 : 6-Xylyloxy)ethyl bromide.* Liquid, b.p. 123°/10 mm. $[n]_D^{30}$ 1.5391. (Found: C, 53.0; H, 5.9; Br, 32.3. $C_{10}H_{13}OBr$ requires C, 52.4; H, 5.7; Br, 34.8%.)

(b) *β(2 : 4 : 6-Mesityloxy)ethyl bromide.* Liquid, b.p. 148°/15 mm. $[n]_D^{30}$ 1.5348. (Found: C, 54.5; H, 6.1; Br, 32.8. $C_{11}H_{15}OBr$ requires C, 54.3; H, 6.2; Br, 32.9%.)

2. Tertiary amines were prepared by allowing the aryloxy ethyl bromide to react in a sealed ampoule at room temperature with an excess of an ethereal solution of dimethylamine or diethylamine. The following were prepared:

(a) *β(2 : 6-Xylyloxy)ethyldimethylamine.* Liquid, b.p. 124°/10 mm. The hydrobromide (TTM.10) crystallized from methanol in needles, m.p. 166°. (Found: C, 52.3; H, 7.2; N, 5.1; Br, 28.8. $C_{12}H_{20}ONBr$ requires C, 52.6; H, 7.3; N, 5.1; Br, 29.1%.)

(b) *β(2 : 6-Xylyloxy)ethyldiethylamine.* Liquid, b.p. 131°/10 mm. The hydrobromide (TTE.10) crystallized from methanol in needles, m.p. 151°. (Found: C, 55.8; H, 8.1; N, 4.6; Br, 26.3. $C_{14}H_{24}ONBr$ requires C, 55.6; H, 8.0; N, 4.6; Br, 26.4%.)

3. Quaternary ammonium salts were prepared as previously described (Hey, 1952). The following are recorded:

(a) *Choline 2 : 6-xylyl ether bromide* (TM.10). Prisms from acetone-methanol, m.p. 209°. (Found: C, 54.0; H, 7.7; N, 4.6; Br, 27.9. $C_{13}H_{22}ONBr$ requires C, 54.2; H, 7.7; N, 4.9; Br, 27.7%.)

(b) *Choline 2 : 4 : 6-mesitylether bromide* (TM.17). Prisms from acetone-methanol, m.p. 186°. (Found: C, 54.5; H, 8.2; N, 4.5; Br, 26.1. $C_{14}H_{24}ONBr$ requires C, 55.6; H, 8.0; N, 4.6; Br, 26.4%.)

(c) *β(2 : 6-Xylyloxy)ethyltriethylammonium bromide* (TE.10). Prisms from acetone-methanol, m.p. 181°. (Found: C, 57.8; H, 8.6; N, 4.3; Br, 23.8. $C_{16}H_{28}ONBr$ requires C, 58.2; H, 8.5; N, 4.2; Br, 24.2%.)

The choline *o*-tolyl ether (TM.18) was prepared as described by Goldfarb (1941).

Summary

1. Choline 2 : 6-xylyl ether bromide has a powerful and long-lasting local anaesthetic effect. It suppresses conduction for long periods of time in postganglionic fibres arising in the superior cervical ganglion of the cat.

2. Certain related compounds have pronounced local anaesthetic activity.

We are indebted to Mrs. Y. Richards for the microanalyses.

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POTENTIATION OF THE PRESSOR ACTION OF NORADRENALINE BY HEXAMETHONIUM, TETRAETHYLAMMONIUM, AND METHANTHELINE

BY

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The pressor action of intravenous adrenaline, noradrenaline, angiotonin, and renin is greatly increased after ganglionic transmission is blocked by tetraethylammonium (Corcoran and Page, 1947; Page and Taylor, 1947, 1950; Moe, 1947; Page, 1949). This pharmacological effect provides a useful clinical tool in the diagnosis of phaeochromocytoma and in the therapy of the serious hypotensive conditions that can follow the administration of ganglion-blocking drugs (La Due, Murison, and Pack, 1948).

The phenomenon has been explained in different and sometimes opposite ways. It was ascribed to a peripheral sensitization by chemical denervation, or to an anti-amine-oxidase activity of tetraethylammonium in the tissues (Page and Taylor, 1947). Later on, the same authors (Page, 1949; Page and Taylor, 1950) considered as a mechanism the release from the liver of a noradrenaline-like substance. The effect could not be produced when the spinal cord segments lower than C6 were destroyed, but it was still present when the spinal cord was simply transected at a high level (Page, 1949; Page and Taylor, 1950).

The latter observation was confirmed by Saint Clair and Stone (1951) and is inconsistent with the hypothesis, suggested by Moe (1947), that the potentiation is due to paralysis of the normal nervous mechanism compensating any haemodynamic modification. Were this hypothesis true, the simple severance of the spinal cord at C1 should prevent the appearance of the potentiation.

The potentiation occurs also after vagotomy or atropine administration. This observation does not support the hypothesis of Rosa and Grassi (1949–50) that the potentiation is due to the blockade of a parasympathetic reflex vasodilatation.

Our experiments were directed toward a further investigation of this controversial problem. We used different drugs and tested them in relation to a single vasoactive substance, (−)-noradrenaline. Besides tetraethylammonium, we have studied hexamethonium, which is without some of tetraethylammonium's secondary effects, and methantheline (" Banthine "), since after its administration the parasympathetic blockade occurs at an earlier stage than the sympathetic.

METHODS

Fifty-six adult dogs anaesthetized with chloralose (70–100 mg./kg.) were used. Blood pressure was recorded from a carotid or femoral artery by a Hg manometer. To obtain a steady hypertension, (−)-noradrenaline (8 to 24 mg./l.) was infused intravenously, at a rate differing from experiment to experiment and dependent on the hypertensive level desired. This hypertension corresponds haemodynamically to the pressor outbursts of phaeochromocytoma. When the blood pressure had reached steady values, ganglion-blocking drugs were injected intravenously.

The following substances were used: hexamethonium bitartrate (0.05 to 6.0 mg./kg.), tetraethylammonium bromide (5.0 to 18.0 mg./kg.), and methantheline bromide (0.06 to 7.0 mg./kg.). Hexamethonium doses are all given in terms of the base, tetraethylammonium and methantheline doses in terms of the salts.

RESULTS

Potentiation of Noradrenaline Hypertension.—Thirty of a total of 31 dogs were subjected to bilateral cervical vagotomy before testing the vascular responses; hexamethonium was used in 15 animals, methantheline in 8, and tetraethylammonium in 8.

In 12 dogs, during an infusion of noradrenaline, an increase of blood pressure occurred after administration of hexamethonium in amounts larger than 2.5 mg./kg.; however 4.0 mg./kg. or more was often required to obtain more constant and clear-cut pressor effects (up to 50–60 mm. Hg).

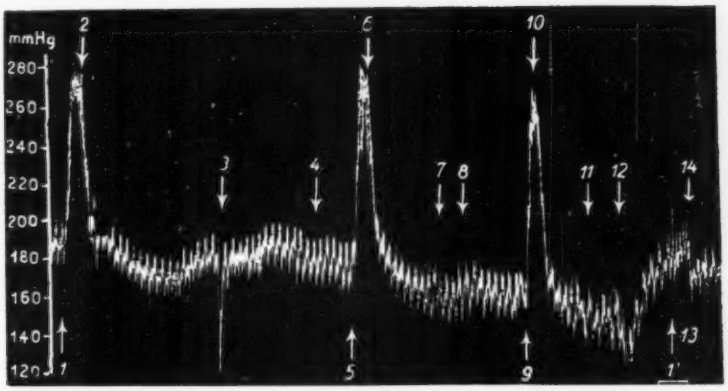

FIG. 1.—Dog, chloralose, vagi cut. Record of arterial blood pressure. Methantheline bromide, i.v., 0.06 mg./kg. at 4, 0.25 mg./kg. at 8, and 3.0 mg./kg. at 12. Peripheral end of right vagus stimulated at 3, 7, and 11. Carotid arteries occluded between 1–2, 5–6, 9–10, and 13–14. (−)-Noradrenaline was infused i.v. to produce a sustained rise of blood pressure throughout the experiment.

The potentiation could be produced several times in the same animal by injecting the effective doses, even at short intervals of time. Doses less than 2.0 mg./kg. did not produce potentiation, even when administered in close succession, but the phenomenon could be observed if the doses were progressively increased. In 3 animals no potentiation was obtained; in 2 of them the drug was administered in insufficient amount, whereas in the third experiment the effect did not occur in spite of large doses and repeated administration.

In 8 vagotomized animals, in which methantheline was injected, the pressor effect of noradrenaline was clearly augmented. The potentiation developed often, but not constantly, after a short-lasting hypotension (Fig. 1). The threshold effect was obtained with 3.0 mg./kg. of the drug. Further increase of blood pressure could be elicited by repeated administration of effective doses, even when a following injection occurred shortly after a previous one (Fig. 2). Subthreshold doses were ineffective even when administered repeatedly.

In 6 to 8 animals, tetraethylammonium bromide potentiated the noradrenaline hypertension. As for hexamethonium and methantheline, repeated administration of effective doses (5.0 to 18.0 mg./kg.) elicited a further hypertension, whereas the close repetition of subthreshold amounts was constantly ineffective. The potentiation could not be observed in two experiments, in spite of the injection of high doses of the drug, which usually produce the phenomenon in other animals.

Thus the three substances studied potentiate the pressor effect of noradrenaline. Generally, the stronger the pressor effect the longer is its duration. The maximal duration was 4–5 min. for tetraethylammonium and hexamethonium, and 7 min. for methantheline. We gained the impression that the magnitude of the potentiation does not depend on the hypertensive level established by infusion of noradrenaline. Of course, this holds only within a given range of pressure values, that is when the peripheral blood vessels can respond to the drug with a further constriction.

Baroceptive Mechanisms.—The potentiation could be ascribed to the suppression, by the drugs, of the baroceptive reflexes which counteract the peripheral vasopressive influence of noradrenaline. A blockade of the vagal cardioinhibitory pathways, or of the efferent autonomic paths regulating the vasomotor tone, is likely to prevent the regulatory influence of both carotid sinus and cardio-aortic reflexes. That the first mechanism alone is not responsible for the potentiation is shown by the well-known fact, which we have been able to confirm in our experiments, that potentiation is still obtainable in bilaterally vagotomized animals. The second mechanism is supported by the fact that the increase of blood pressure elicited by bilateral clamping of the common carotid (the so-called carotid sinus reflex) is

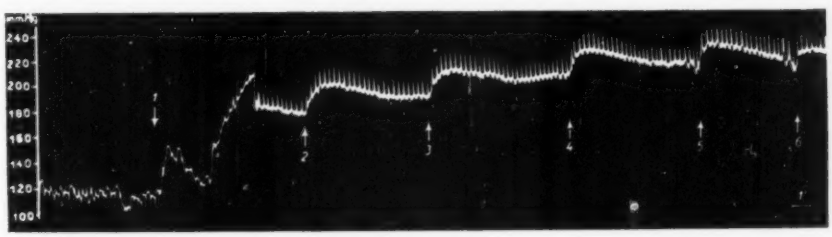

FIG. 2.—Dog, chloralose, vagi cut. (−)-Noradrenaline infused continuously i.v. at rate of 3.4 mg./kg./min. from 1. At 2, 4, 5, and 6, 6.0 mg./kg. methantheline bromide i.v., and at 3, 4.0 mg./kg. methantheline bromide i.v.

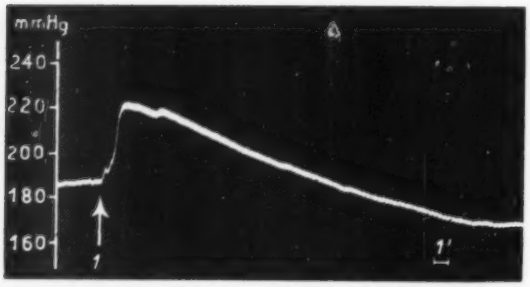

FIG. 3.—Dog, chloralose, vagi cut and spinal cord ablated from C1 to L2. Continuous i.v. (−)-noradrenaline, at a rate of 5.0 mg./kg./min. At 1, tetraethylammonium bromide 15.0 mg./kg. i.v.

completely abolished by these drugs and with the same doses which give potentiation. Actually, as soon as the influence of the carotid sinus baroceptors on the vasomotor component of the circulation is abolished, the potentiation is observed. These observations seem to support the hypothesis that blockade of baroceptive regulation and potentiation are causally related phenomena.

Serious difficulties are, however, shown by our methantheline experiments. With this drug, the fall of blood pressure elicited by faradizing the

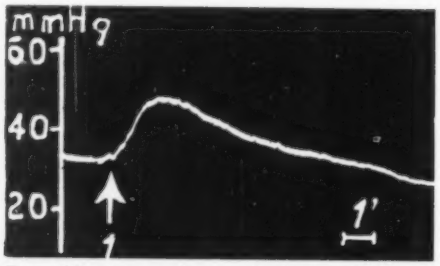

FIG. 4.—Dog, chloralose, vagi cut and spinal cord ablated from C1 to L2. Noradrenaline was *not* infused. At 1, tetraethylammonium bromide 15.0 mg./kg. i.v.

peripheral end of the vagus nerve is blocked for doses (0.06 mg./kg.) about 50 times smaller than those which are necessary for blocking the carotid sinus reflexes (Fig. 1). And yet the potentiation is obtained only when these reflexes are abolished (Fig. 1, observations 12, 13, and 14). Hence there is no doubt that potentiation is not due to blockade of the cardio-inhibitory component of the baroceptive reflexes. A vasomotor compensation by these reflexes of the peripheral action of noradrenaline might occur either through an increase of vasodilator activity or through a lowering of vasoconstrictor tone. It is unlikely that, for doses of methantheline about 50 times higher than those which block the vagal inhibition, the parasympathetic pathways are still active. If this assumption, which, however, cannot be regarded as crucially proved, is accepted, the conclusion should be drawn that the drug acts by preventing the peripheral effect of a baroceptive decrease of the central vasomotor tone. This appears to be an unlikely assumption, however, since the peripheral vasomotor tone was controlled by the continuous noradrenaline infusion, and is disproved, moreover, by the experiments reported below.

Supraspinal and Spinal Mechanisms.—If the suppression of the normal nervous mechanisms of circulatory homeostasis were the cause of potentiation, this would be absent in the spinal preparation after bilateral vagotomy. That this is not so, at least for tetraethylammonium, has been clearly demonstrated by Page and Taylor (1950) and Saint Clair and Stone (1951), who observed an increased pressor effect of noradrenaline injections following administration of tetraethylammonium in both normal and spinal animals. However, it is well known that tetraethylammonium has, besides a ganglion-blocking action, a pressor effect the origin of which is not clear.

It appeared worth while, therefore, to test the effect also of hexamethonium and methantheline in spinal (section at C1) vagotomized dogs, during continuous noradrenaline infusion. In 7 spinal dogs the potentiation occurred at the same doses, and with the same duration and intensity, as in normal animals. The phenomenon was not modified by the integrity or the section of the vagi.

To test the importance of the spinal cord in potentiation, we have carried out total ablation of the spinal cord, from C1 to L2, in 12 vagotomized animals. In 5 of these, to which tetraethyl-

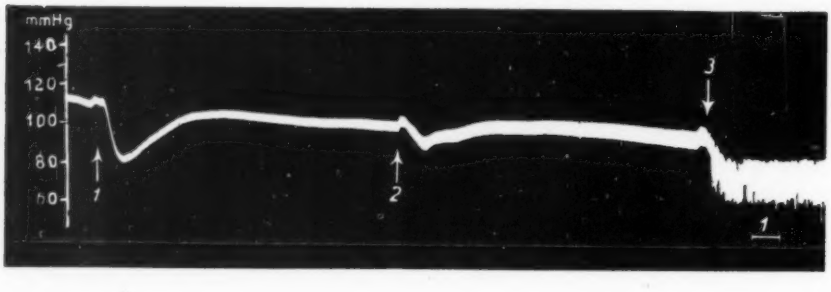

FIG. 5.—Dog, chloralose, vagi cut and spinal cord ablated from C1 to L2. Moderate hypertension was caused by continuous intravenous infusion of (−)-noradrenaline. At 1, 2, and 3, hexamethonium bitartrate 5.0 mg./kg. i.v.

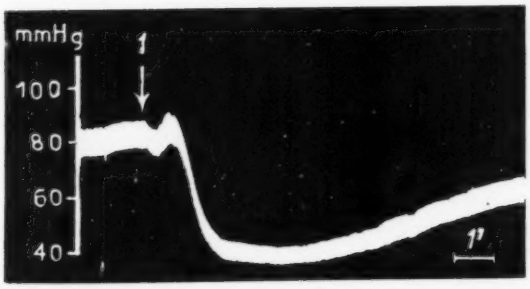

FIG. 6.—Dog, preparation as in Fig. 5. At 1, 4.0 mg./kg. methantheline bromide i.v.

ammonium was injected, a constant hypertensive effect was obtained (Fig. 3), which, however, occurred even when no noradrenaline was infused (Fig. 4). In the other 7 experiments, in which hexamethonium and methantheline were used, the administration of these drugs constantly elicited a blood-pressure decrease. Hence, the potentiation by these drugs was abolished by ablation of the cord (Figs. 5 and 6).

DISCUSSION

The potentiation of the pressor effect of noradrenaline by the injection of tetraethylammonium (Corcoran and Page, 1947 ; Page and Taylor, 1947 and 1950 ; Moe, 1947 ; Page, 1949) and of hexamethonium (Paton, 1951) is confirmed and the same phenomenon can be elicited also by administration of methantheline.

Our experimental methods have differed from those used by most previous authors. They tested the effect of noradrenaline in normal conditions and after intravenous administration of tetraethylammonium. The results obtained by the latter method are open to three main criticisms: (i) data obtained in two different haemodynamic conditions (normal pressure and hypotension from ganglionic blockade) are compared ; (ii) the augmented pressor responses to noradrenaline could be simply a consequence of the lower blood-pressure level produced by injection of the blocking drug (this objection could be safely dismissed only if the absolute level of noradrenaline hypertension, after administration of ganglion-blocking drugs, were higher than in normal conditions) ; (iii) data obtained at some interval of time are compared, when various factors may have interfered with the effect. It is well known, for example, that the action of ganglion-blocking substances is subject to rapid modifications.

Our experimental procedure overcomes these objections, emphasizes the critical importance of the dose of the ganglion-blocking agent and, in particular, enables the production of closely succeeding, and even superimposed, potentiations. Furthermore, interruption of noradrenaline infusion in the animals with the spinal cord destroyed from C1 to L2 has provided clear evidence for a direct hypertensive action of tetraethylammonium. This effect had been observed by previous authors (Page, 1949). Finally, since hexamethonium and methantheline have not been shown to share such an action, the potentiation can be ascribed to the main common effect of the three substances—the ganglion-blocking activity. However, the use of tetraethylammonium in the study of the potentiation does not appear completely safe, because of its hypertensive side-effect. This is not likely to depend on an inhibition of amine oxidase, because it occurs, without infusion of noradrenaline, after the spinal cord is destroyed.

The evidence provided in our experiments does not support the hypothesis advanced by Moe (1947), that the potentiation is a consequence of the paralysis of the brain stem mechanisms concerned in circulatory homeostasis. Since the effect was abolished by ablating the spinal cord, from C1 to L2, these segments seem to be essential for the phenomenon of potentiation.

Only tetraethylammonium shows a hypertensive effect in animals with the spinal cord ablated, but this occurs without infusion of noradrenaline, and, therefore, it cannot be considered a potentiation. On the other hand, the hypothesis of a decreased vascular sensitivity in such animals, as a result of surgical shock, can be dismissed, since in the same animals very good responses were elicited by noradrenaline infusion.

The potentiation might occur in the spinal animal either by abolishing its vasodilator tone or by stimulating the preganglionic sympathetic neurones which are responsible for the spinal vasoconstrictor tone (Heymans *et al.*, 1936a, b, c ; Leriche and Fontaine, 1930 ; Bartorelli and Capretti, 1946).

The first hypothesis is only indirectly supported by Uvnäs' experiments, since we do not know if the cholinergic vasodilator outflow reported by the Swedish investigators is tonic in character.

We are at loss to understand how a stimulation of sympathetic preganglionic neurones (second hypothesis) might be effective, since the potentiation occurs, as shown by our carotid sinus experiments, just when the main efferent vasopressor paths are blocked.

It could be maintained that the spinal cord is actually not involved in the potentiation, which might be explained in terms of some purely peripheral effect such as a blockade of amine oxidase.

It must be conceded that when the spinal cord is transected at C1, or ablated, the production of noradrenaline and adrenaline, both as hormones and as transmitters, is likely to be so low that the influence of a blockade of the amine oxidase might easily be missed. It is difficult to understand, however, why injected noradrenaline should not be potentiated in these conditions. Our experiments have shown that in the preparations with the spinal cord ablated, and infused with noradrenaline, potentiation never occurs, and that a fall of blood pressure is elicited by injection of methantheline and hexamethonium. Further experiments are obviously required before a satisfactory explanation of the mechanism of potentiation can be given.

Our procedure is the experimental counterpart of the pathogenic and haemodynamic conditions associated with a phaeochromocytoma. Injections of ganglion-blocking drugs are used as a diagnostic test in man, a hypertensive response being considered as a positive sign of phaeochromocytoma. However, the results of the present experiments indicate that the potentiation occurs on administration of ganglion-blocking drugs in amounts far larger than those used in man for clinical purposes. Hence, the diagnostic value of the test is doubtful, unless it can be demonstrated that the sensitivity to ganglion-blocking agents is greater in man than in dogs, or that it is decreased by anaesthesia. Furthermore, the potentiation occurs only in the presence of a considerable amount of circulating noradrenaline. It would be advisable, therefore, to time the test during typical hypertensive outbursts, but this procedure would produce at least a temporary increase of the patient's subjective disturbances.

It might be convenient to aim the test at producing evidence not of a further hypertension but of the absence of hypotension. However, it has to be remembered that some hypertensive patients, without any adrenal medullary tumour, do not show hypotensive responses to the administration of ganglion-blocking drugs.

On the other hand, the use of the high doses which were effective in our experimental animals would expose the patient to the danger of an enormous fall of blood pressure, whenever the hypertensive condition was not due to a phaeochromocytoma. Serious danger could be avoided, however, if a noradrenaline solution were on hand to be infused intravenously (Bartorelli and Folli, 1954). We do not share the opinion of those authors (Page and Taylor, 1947) who think that the use of noradrenaline during deep hypotension from ganglion-blockade is contraindicated. We think that continuous intravenous infusion, drop by drop, with frequent blood-pressure measurements, permits the exact adjustment of the dose to the requirements of the patient ; the necessary amount of noradrenaline is decreased by the potentiating effect of the ganglion-blocking drug.

Summary

1. The intravenous injection of hexamethonium (2.5 to 6.0 mg./kg.), methantheline (3.0 mg./kg. or more) and tetraethylammonium (5.0 to 18.0 mg./kg.) elicits an increase of blood pressure in animals into which (−)-noradrenaline is continuously infused.

2. The potentiation is not affected by vagotomy and section of the spinal cord at a high level.

3. The potentiation occurs only when both parasympathetic and sympathetic transmissions are blocked. Small doses of methantheline, which block only the parasympathetic innervation of the heart, are insufficient to produce potentiation.

4. Ablation of the spinal cord (from C1 to L2) prevents the potentiation ; in these conditions tetraethylammonium still causes a rise of blood pressure, whether (−)-noradrenaline is infused or not. On the contrary, no blood-pressure increase results from the injection of hexamethonium and methantheline in such animals.

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THE ACTION OF HISTAMINE AND PILOCARPINE ON THE SUPERIOR CERVICAL GANGLION AND THE ADRENAL GLANDS OF THE CAT

BY

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Dale and Laidlaw (1912) demonstrated stimulation of the adrenal glands of the cat by pilocarpine, and Burn and Dale (1926) observed it after the injection of histamine. This action on the adrenal glands by both histamine and pilocarpine is partly responsible for the contractions of the normal and denervated nictitating membranes of the intact cat. But another factor seems to be involved in the response of the membranes, as Burn and Trendelenburg (1954) observed that sometimes the normal membrane responded more than the denervated membrane and that in some experiments the membranes responded after adrenalectomy. By perfusing the head of the cat with a Locke-dextran solution they demonstrated that both histamine and pilocarpine had no direct action on the nictitating membranes. The superior cervical ganglion of the normal side was not removed in their experiments on the whole animal and it thus seemed possible that these substances stimulated the ganglion.

Dale and Laidlaw (1912) demonstrated the stimulation of the superior cervical ganglion of the cat by pilocarpine in the whole cat, and Ambache (1949) confirmed this in the perfused preparation. Histamine, on the other hand, was found by Feldberg and Vartiainen (1935) not to stimulate the perfused superior cervical ganglion, and Konzett (1952) confirmed this for most of his experiments. He observed, however, a stimulation of the ganglion in 4 out of 22 preparations.

These mostly negative findings obtained with perfused preparations stand in contrast to the ability of histamine to stimulate the adrenal glands which are left in the normal circulation (Szczygielski, 1932). It was therefore decided to re-investigate the question of a stimulation of sympathetic ganglia by histamine and pilocarpine, using preparations in which the normal blood supply of the superior cervical ganglion was not interfered with.

METHODS

Cats of 2 to 4 kg. were used. After inducing anaesthesia with ether, 80 mg./kg. chloralose was injected intravenously. Intra-arterial injections were made into the central end of the lingual artery while occluding the external carotid artery. Thus the injected substance was diverted towards the superior cervical ganglion. With the exception of the internal carotid artery which was occasionally found and tied, no further attempts at isolation were made.

The contractions of the nictitating membrane (and in some experiments of both membranes) were recorded with an isotonic lever fitted with a frontal writing point magnifying the movements of the membrane 7.5 times. The cervical sympathetic chain was cut. For preganglionic stimulation it was placed on a pair of shielded electrodes and covered with warm liquid paraffin. The stimulus had a frequency of 17/sec. and a duration of 0.7 msec. The blood pressure was recorded from either the carotid artery of the other side or from the femoral artery.

In some experiments spinal preparations were used, set up as described by Burn and Trendelenburg (1954). As the results with these preparations did not differ from those obtained under chloralose anaesthesia, they will not be mentioned separately.

For arterial injection to adrenal glands, cats under chloralose anaesthesia were eviscerated and a cannula was tied into the central end of the coeliac artery. The aorta was tied below the kidneys. The blood pressure was recorded from the carotid artery.

The following substances were used: histamine dihydrochloride or histamine acid phosphate, dissolved in saline and neutralized with N/10 NaOH, the dose being expressed as free base; pilocarpine nitrate, the dose being expressed as free base; hexamethonium bromide, nicotine hydrogen tartrate, cocaine hydrochloride, atropine sulphate, mepyramine maleate, all expressed as salts.

RESULTS

Intravenous Injections

Stimulation of the superior cervical ganglion of a cat under chloralose anaesthesia by intravenous injection of histamine is demonstrated in Fig. 1,

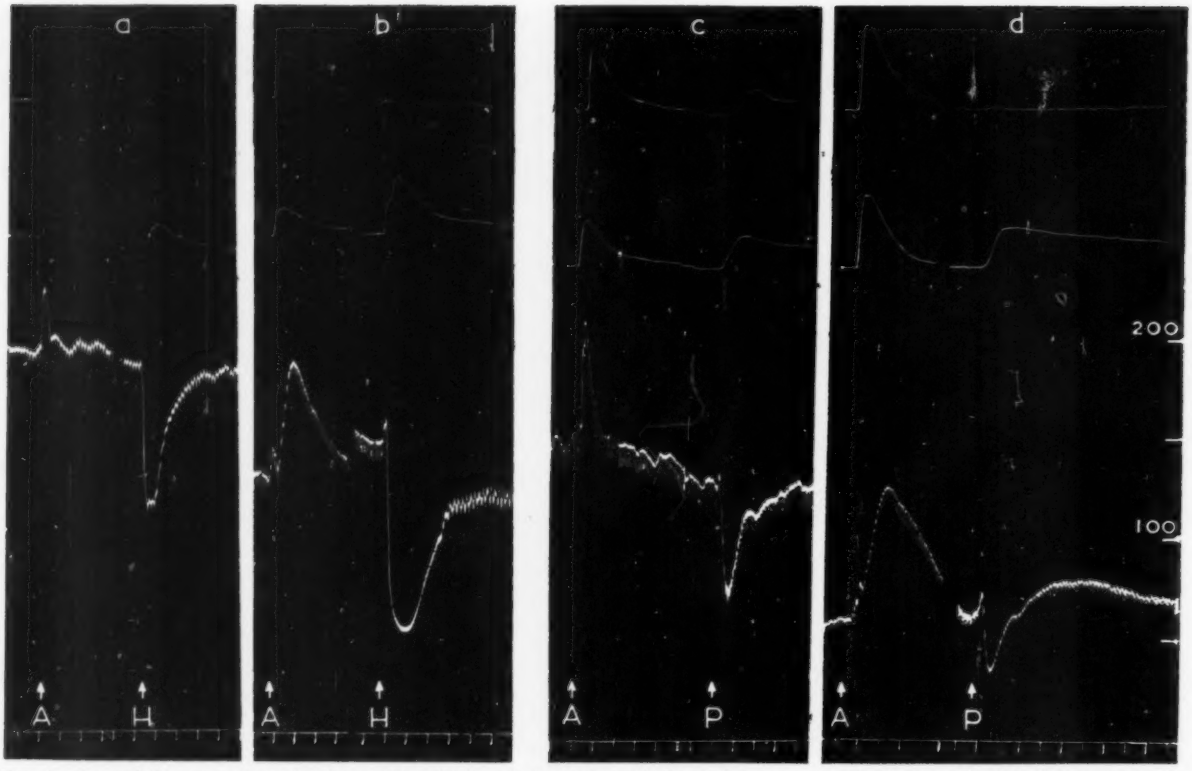

FIG. 1.—(*a*) and (*b*), cat under chloralose; (*c*) and (*d*), spinal cat. Records from above are: acutely denervated nictitating membrane; normal nictitating membrane with its preganglionic fibres cut; arterial blood pressure; time in 30 sec. Intravenous injections were: A, 5 μg. adrenaline; H, in (*a*) 65 μg. histamine; in (*b*), 130 μg. histamine; P, 150 μg. pilocarpine. Adrenalectomy and evisceration between (*a*) and (*b*) and between (*c*) and (*d*). Note stronger contraction of normal side where the ganglion was left intact.

sections (a) and (b). Throughout the experiment injections of 5 μg. adrenaline (A) were given as a control. Section (a) shows equal contractions of the acutely denervated and the normal nictitating membrane elicited by the injection of 65 μg. histamine at H ; section (b) the effect of twice that dose after adrenalectomy and evisceration: the denervated membrane no longer contracted, whereas a large contraction was elicited on the innervated side. The preganglionic fibres of the innervated side were cut in order to exclude central impulses.

Sections (c) and (d) show similar results on a spinal cat given 150 μg. pilocarpine at P intravenously, (c) before, and (d) after adrenalectomy and evisceration.

Intra-arterial Injections

To obtain stimulation of the superior cervical ganglion by intravenous injections, large amounts of histamine were required, and from these recovery of the blood pressure and of sensitivity of the ganglion was slow. The method of intra-arterial injection into the lingual artery was therefore adopted for the following experiments.

Intra-arterial injections of 2–55 μg. histamine (using histamine dihydrochloride in most of the experiments), dissolved in 0.2 to 0.3 ml. of saline and neutralized, caused a contraction of the nictitating membrane in 23 out of 28 cats. The usual dose was 15–20 μg. histamine and contractions of the membrane from 4 to 45 mm. (on the drum) were recorded. Increasing responses were observed with increasing doses. It was found that desensitization regularly occurred when injections were repeated. Care was therefore taken in each experiment to find the suitable interval between injections and to keep this interval constant. It varied from 5 to 30 min.

Intra-arterial injections of 25–40 μg. pilocarpine were found to stimulate the superior cervical ganglion in 6 out of 10 cats. The responses were very similar to those produced by histamine, but they were of longer duration (see Figs. 1 and 2) and desensitization was still more marked ; time intervals from 20–40 min. between successive injections were necessary.

As it was observed that the latent period between the intra-arterial injection and the response of the

membrane was considerably longer than that observed after intra-arterial injection of acetylcholine or nicotine, it was necessary to show that this response of the membrane was of ganglionic origin. There were three possible sources of error: (1) the stimulation of the ganglion was unspecific; (2) the contraction of the membrane was due to stimulation of the adrenal glands; and (3) the substances had an action on the membrane itself. In order to exclude these possibilities the following experiments were carried out.

(1) Control injections of saline into the lingual artery were without any effect. All solutions were carefully neutralized and the use of histamine dihydrochloride excluded a stimulation of the ganglion by the phosphate present in histamine acid phosphate.

(2) When comparing the effect of intra-arterial with intravenous injections of equal amounts of histamine or pilocarpine, it was found that up to 50 μg. of these substances injected intravenously had very little or no effect at all on the nictitating membrane (Fig. 2). Adrenalectomy did not modify the response to intra-arterial histamine or pilocarpine if the injection was made rather soon after the operation. When recording both membranes it was observed that only the membrane of the injected side contracted. Removal of the superior cervical ganglion or cutting of the post-

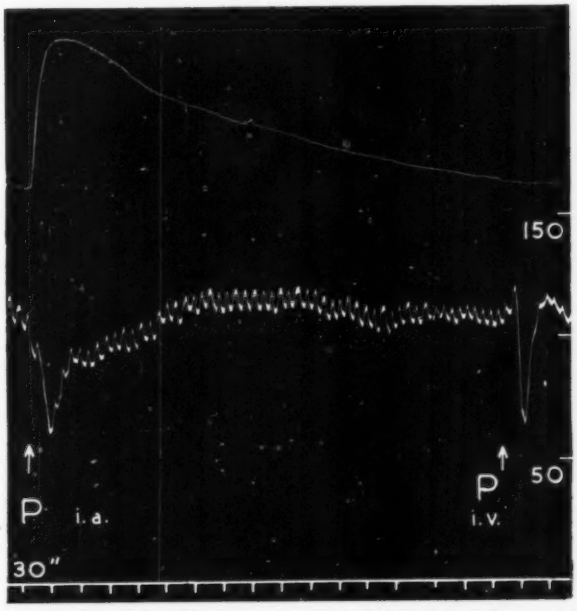

FIG. 2.—Cat under chloralose. Normal nictitating membrane and arterial blood pressure. Injection of 40 μg. pilocarpine (P) into the lingual artery during occlusion of the external carotid artery (i.a.) and intravenously (i.v.). Note duration of ganglionic response and absence of response to intravenous injection.

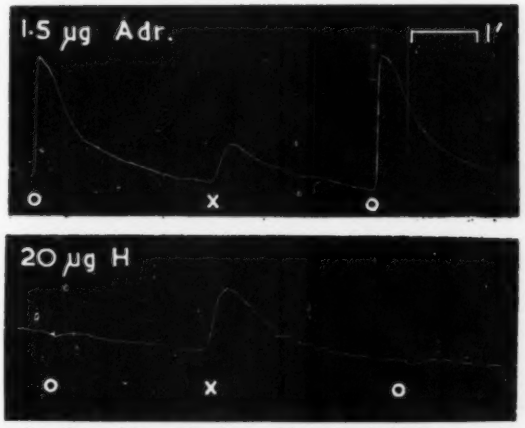

FIG. 3.—Cat under chloralose. Normal nictitating membrane. Upper record: injection of 1.5 μg. adrenaline into the lingual artery without occlusion (o) and during occlusion (×) of the external carotid artery. Lower record: injection of 20 μg. histamine into the lingual artery without occlusion (o) and during occlusion (×) of the external carotid artery. Note the direct action of adrenaline on nictitating membrane in contrast to ganglionic stimulation by histamine.

ganglionic fibres abolished the response to intra-arterial injections of histamine or pilocarpine.

(3) As no attempt was made to isolate the superior cervical ganglion, the injected histamine or pilocarpine might act directly on the nictitating membrane. Burn and Trendelenburg (1954) demonstrated that both substances were unable to contract the nictitating membrane in a perfused preparation. In order to confirm this for membranes the circulation of which was left undisturbed, the following experiment was performed. An injection of 20 μg. histamine was made into the lingual artery (a) when the external carotid artery was occluded and (b) when it remained open. The injection caused a contraction of the nictitating membrane when injected during occlusion of the external carotid artery, but not when it was unoccluded, as Fig. 3 shows. Adrenaline, on the other hand, readily elicited a contraction in as low a dose as 1.5 μg. when the external carotid artery was open, but had a much smaller action during occlusion. Pilocarpine was found to have similar actions to those of histamine, thus confirming earlier observations.

These findings indicate that the contraction observed after intra-arterial injection of a neutralized solution of histamine or pilocarpine was due to stimulation of the superior cervical ganglion.

Hexamethonium.—Intravenous injections of up to 20 mg. hexamethonium were repeatedly found to have no effect on the ganglionic response to histamine or pilocarpine, or to reduce it but slightly. One experiment, shown in Fig. 4, may illustrate this. After two control injections of

20 μg. histamine the cervical sympathetic chain was stimulated and the strength of the stimulus was chosen so as to give a contraction of approximately the same height. When 5 mg. hexamethonium was injected intravenously the effect of pre-

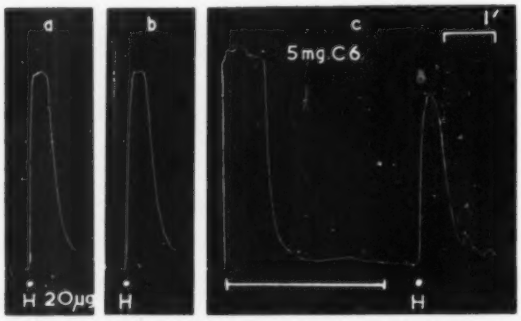

FIG. 4.—Cat under chloralose. Normal nictitating membrane Injection of 20 μg. histamine into the lingual artery during occlusion of the external carotid artery (H). 20 min. interval between (*a*) and (*b*) and (*b*) and (*c*). In (*c*) submaximal preganglionic stimulation (——) and i.v. injection of 5 mg. hexamethonium bromide. Note that response to histamine was not much affected by hexamethonium in a dose which blocked transmission.

ganglionic stimulation was abolished completely, but the response to 20 μg. histamine was only slightly depressed. Similar experiments were carried out with pilocarpine and similar results were obtained. Control experiments showed that in atropinized cats these doses of hexamethonium regularly abolished the response to intra-arterial injections of 25–100 μg. acetylcholine. Thus it was found that hexamethonium in doses which blocked both transmission and injected acetylcholine had but a very slight effect on the response to histamine and pilocarpine.

Nicotine.—In some experiments nicotine was given intravenously, paralysing ganglia by successive injections of increasing doses (0.5 to 10 mg.) and repeating the last dose until there was neither a response of the nictitating membrane nor a rise of blood pressure. Histamine or pilocarpine then injected intra-arterially into the lingual artery always failed to stimulate the superior cervical ganglion. In other experiments 6 to 8 injections of 50 μg. nicotine each were made into the lingual artery within 2–3 min. There was a response of the ganglion to the first three injections, the ganglion then being paralysed. The next injection of histamine or pilocarpine always failed to stimulate the superior cervical ganglion and partial recovery was observed 30 min. later. In some experiments it was observed that 30–60 min. after nicotine the response to histamine or pilocarpine was much larger than that to the control injections before any nicotine had been given.

Cocaine.—This substance was found to reduce the action of histamine and pilocarpine on the superior cervical ganglion, as Fig. 5 illustrates. After 2 control injections of 20 μg. histamine (sections (a) and (b)) preganglionic stimulation

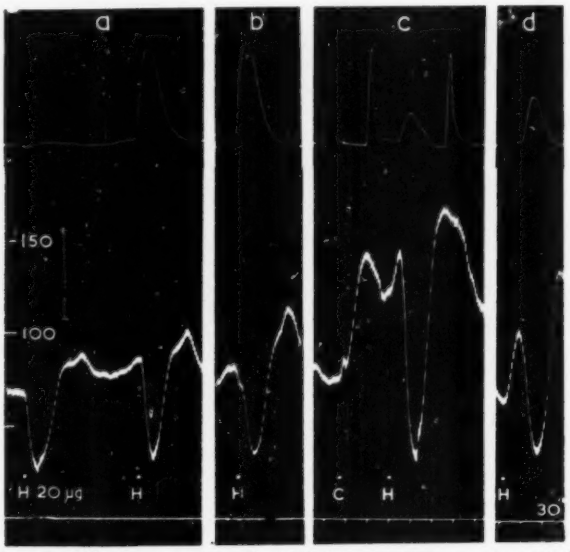

FIG. 5.—Spinal cat. Normal nictitating membrane and arterial blood pressure. (*a*) Injection of 20 μg. histamine into the lingual artery, first without and then with occlusion of external carotid artery. (*b*) As second injection in (*a*) after 20 min., (*c*) 20 min. later: 5 sec. of submaximal preganglionic stimulation, then i.v. injection of 0.5 mg. cocaine hydrochloride, followed by preganglionic stimulation, injection of 20 μg. histamine into the lingual artery and preganglionic stimulation. (*d*) 20 min. later. Note that the response to histamine was reduced by an amount of cocaine which did not affect preganglionic stimulation.

was applied for 5 sec., the strength of stimulus being chosen so as to give a submaximal contraction of similar height. After intravenous injection of 0.5 mg. cocaine the response to preganglionic stimulation was unaltered whereas the response to histamine was much reduced. Partial recovery was observed after 18 min. There was no diminution of the fall of blood pressure after histamine. In another experiment the intramuscular injection of 20 mg. cocaine abolished the response to 2 μg. histamine for more than 4 hr., 20 μg. histamine starting to elicit a response after 160 min. Furthermore it was found that the ganglionic response to pilocarpine was also reduced by cocaine in doses which did not interfere with the response to preganglionic stimulation. Control experiments showed that in atropinized cats small amounts of cocaine did not depress the response to intra-arterial injections of acetylcholine whereas 2.5 mg. cocaine or more potentiated the response.

Mepyramine.—Increasing amounts of the antihistamine substance mepyramine reduced and

finally abolished the response to 20 μg. histamine, as shown in Fig. 6. Preganglionic stimulation, recorded before and after injection of the highest dose of mepyramine, was not affected. 150 μg. mepyramine was found to block both the ganglionic and the blood-pressure responses to 20 μg. histamine. Recovery was observed after 40 min. but was not complete. Pilocarpine, on the other hand, still exerted its ganglion-stimulating action after 500 μg. mepyramine.

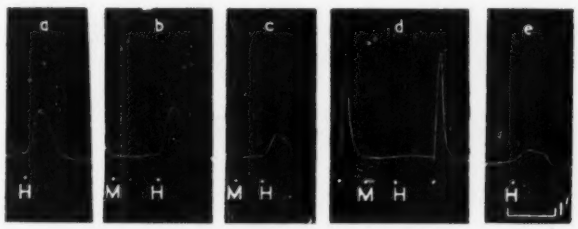

FIG. 6.—Spinal cat. Normal nictitating membrane. H, 20 μg. histamine injected into lingual artery during occlusion of external carotid artery. Time intervals between tracings 20 min. Mepyramine maleate (M) i.v.: 20 μg. in (*b*), 60 μg. in (*c*), and 150 μg. in (*d*). In (*d*) submaximal preganglionic stimulation for 5 sec. before and after injection of mepyramine. Note that an amount of mepyramine which did not affect preganglionic stimulation abolished response to histamine.

Atropine.—This substance in doses up to 2 mg. had no effect on the ganglionic response to histamine, whereas 200 μg. atropine abolished the response to pilocarpine for about 3 hr.

Stimulation of Adrenal Medulla

Injections of 0.13 to 3.5 μg. histamine into the central end of the coeliac artery led to a rise of blood pressure of 15–50 mm. Hg, due to stimulation of the adrenal glands. A constant response was obtained if a time interval of at least 10 min. was allowed between injections. Desensitization was evident with shorter time intervals, the blood-pressure response to a given dose of histamine declining the shorter the interval.

Pilocarpine had a very similar action, but greater amounts were required (7.5–40 μg.). This was not expected, as the ratio of equiactive doses of intralingual injections of histamine and pilocarpine was *ca.* 1:2. These figures were approximate and were obtained from comparisons in several experiments; it was unfortunately not possible to compare the ganglionic effects of both drugs in the same animal. As desensitization was much less pronounced after stimulation of the adrenal glands by histamine or pilocarpine, it was possible to find equiactive doses of these two substances. In 3 cats ratios of 1:57, 1:57 and 1:43 were found, thus showing that pilocarpine was considerably less active on the adrenal glands than histamine.

Hexamethonium.—Hexamethonium in intravenous doses up to 54 mg. increased the pressor response to histamine in proportion as it increased the response to adrenaline or noradrenaline.

Nicotine.—Szczygielski (1932) observed that paralysing doses of nicotine reduced or abolished the pressor rise after intra-arterial injection of histamine. The same was found to be true of pilocarpine, as Fig 7 shows. After 14 intra-arterial injections of 50 μg. nicotine each, the blood-pressure rise after 45 μg. pilocarpine was less than half of the control, full recovery being observed 15 min. later (Fig. 7 (b)).

Cocaine was injected intravenously in doses up to 10 mg., and always potentiated the responses to both histamine and pilocarpine as well as to control injections of adrenaline. There was no sign of a depression of the stimulation of the adrenal glands by histamine or by pilocarpine (Fig. 8). This was also observed in preparations the splanchnic nerves of which had been freshly cut.

Mepyramine, which was found by Emmelin and Muren (1949) to block the action of histamine on the adrenal glands in as low a dose as 10 μg., did not modify the response to pilocarpine. The amount of mepyramine injected intravenously was 300 μg. (Fig. 7 (c)).

Atropine in a dose of 400 μg. abolished the response to pilocarpine (Fig. 7 (d)) without interfering with the action of histamine.

These experiments showed that with one exception there was general agreement between the

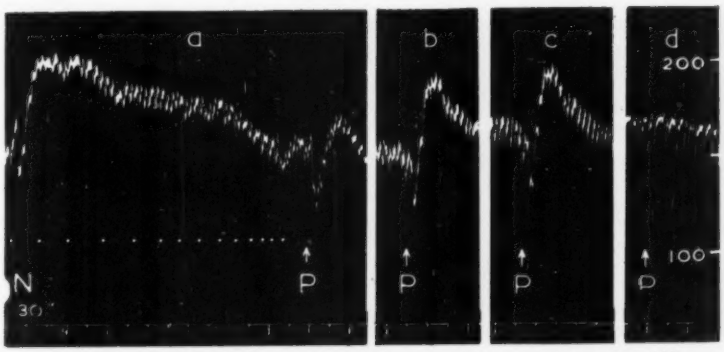

FIG. 7.—Cat under chloralose, eviscerated, and with aorta tied below kidneys. (*a*) 14 injections, each of 50 μg. nicotine tartrate into coeliac artery, followed by intra-arterial injection of 45 μg. pilocarpine (P). (*b*) 15 min. later. (*c*) 15 min. later after 300 μg. mepyramine i.v. (*d*) After 400 μg. atropine sulphate i.v. Note that nicotine reduced the response of the adrenal glands to pilocarpine; that mepyramine did not affect the response, whereas atropine abolished all actions of pilocarpine.

characteristic properties of the superior cervical ganglion and the adrenal glands, as far as the actions of histamine and pilocarpine were concerned. The only substance which exhibited a discrepancy was cocaine, blocking the superior cervical ganglion in very low doses and not modifying the liberation of pressor amines from the adrenal glands by histamine or pilocarpine even after 20-fold doses.

Discussion

The action of histamine and pilocarpine on the nictitating membrane is well known to be due in part to an action on the adrenal glands. The experiments, in which Dale and Laidlaw (1912) demonstrated that pilocarpine had an action on the superior cervical ganglion also, have escaped similar attention. Evidence has now been obtained confirming this action of pilocarpine and showing that histamine also exerts a direct stimulant action on the ganglion. The varying results seen on the nictitating membranes with both these substances (Burn and Trendelenburg, 1954) thus receive an explanation. The frequent observation that the denervated membrane contracts more than the normal membrane may be explained by the release of adrenaline and noradrenaline from the adrenal glands producing a large effect on the denervated membrane, an effect which is increased by cocaine and abolished by adrenalectomy. If, however, the superior cervical ganglion is acutely removed in the course of an experiment, histamine then has been shown to have a greater effect on the innervated side, and this effect persists after adrenalectomy and is abolished by cocaine. It is due to a stimulation of the ganglion itself, since it is seen when small quantities of histamine (or pilocarpine) are injected into the lingual artery during occlusion of the external carotid artery, and it is not seen when the external carotid remains open, leaving a direct pathway to the nictitating membrane.

The actions of histamine and of pilocarpine on the ganglion are similar in being paralysed by excess of nicotine and by cocaine, and in being unaffected by hexamethonium. They are dissimilar in that the action of histamine is blocked by mepyramine but not by atropine, whereas the reverse is true for pilocarpine.

The adrenal glands responded to low doses of histamine and to moderate doses of pilocarpine when these substances were injected into the central end of the coeliac artery after tying the aorta below the kidneys. Desensitization to repeated injections was much less prominent than in the superior cervical ganglion. The only difference between the actions of these substances was quantitative. Whereas they seemed to be roughly equiactive when injected into the lingual artery, pilocarpine was found to have only one-fiftieth of the activity of histamine on the adrenal glands.

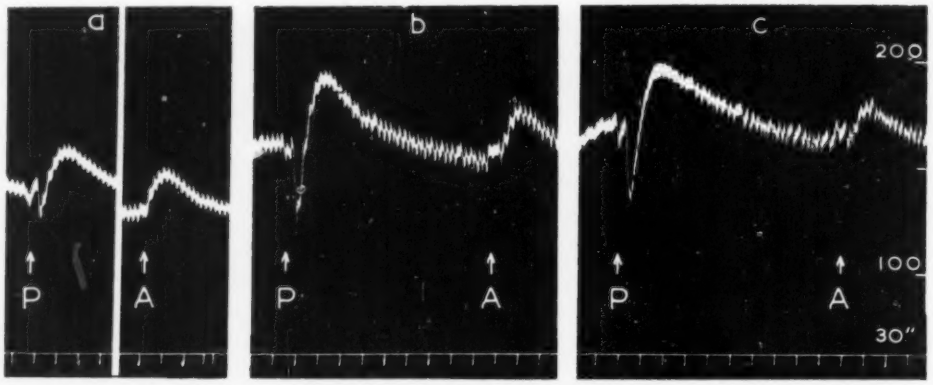

Fig. 8.—Cat under chloralose, eviscerated, and with aorta tied below kidneys. Arterial blood pressure. P, 45 μg. pilocarpine injected into central end of coeliac artery. A, 0.2 μg. adrenaline i.v., (*a*) before, (*b*) after 1 mg. cocaine i.v., and (*c*) after 5 mg. cocaine i.v. Note that ten times more cocaine than used in Fig. 5 did not reduce the response of the adrenal glands to pilocarpine.

The finding that hexamethonium on the superior cervical ganglion readily blocked both transmission and the response to injected acetylcholine without interfering with the response of the superior cervical ganglion or of the adrenals to histamine or pilocarpine indicated that these substances have a direct action on the ganglion cell, acetylcholine not being involved.

Szczygielski (1932) found that the adrenal glands were very sensitive to histamine and that nicotine abolished the response to small doses of histamine. In the present series of experiments it was found that nicotine blocked the effect of histamine and

pilocarpine on the superior cervical ganglion and that it reduced the response of the adrenal glands to intra-arterial injection of pilocarpine.

Cocaine was found to potentiate the blood-pressure rise after injection of either histamine or pilocarpine into the coeliac artery, but to block the action of these substances on the superior cervical ganglion. This blocking action of cocaine needs further investigation.

When comparing the response of the superior cervical ganglion to either histamine or pilocarpine with the stimulation of the ganglion by acetylcholine or nicotine, three observations were consistently made: the latency period between the injection of histamine or pilocarpine and the onset of contraction of the nictitating membrane was longer, and both the contraction and the desensitization were of much longer duration than those observed after approximately equiactive doses of acetylcholine or nicotine.

With our present knowledge it is impossible to answer the question why histamine usually failed to stimulate the perfused superior cervical ganglion (Feldberg and Vartiainen, 1935; Konzett, 1952) while stimulating the ganglia the circulation of which is intact. Whatever the reasons for this discrepancy, histamine must be regarded as a substance able to exert an influence on the autonomic nervous system of the cat. Various adrenaline-like effects of histamine require consideration from this point of view. It is not known whether the liberation of histamine from tissues in the body by drugs or under certain conditions such as anaphylaxis or shock may lead to a change in excitability of the autonomic nervous system, but it is of interest that Chauchard and Mazoué (1954) detected, by measurements of chronaxie, an increased excitability of the sympathetic nervous system during anaphylactic reactions.

Summary

1. By comparing the response of a normal nictitating membrane with that of an acutely denervated one to intravenous injections of histamine or pilocarpine, a stimulation of the superior cervical ganglion was demonstrated in intact and adrenalectomized cats.

2. Intra-arterial injections of histamine or pilocarpine into the lingual artery during occlusion of the external carotid artery were found to stimulate the superior cervical ganglion without interference from unspecific factors or liberation of medullary amines from the adrenal glands.

3. It was confirmed that both histamine and pilocarpine have no direct action on the nictitating membrane.

4. Ganglionic responses to histamine or pilocarpine were found to show rapid desensitization. They were not abolished by doses of hexamethonium which abolished preganglionic stimulation. They were abolished by paralysing doses of nicotine. They were abolished by cocaine in doses which did not interfere with preganglionic stimulation. Atropine specifically inhibited pilocarpine; mepyramine equally specifically inhibited histamine.

5. Injections of histamine or pilocarpine into the central end of the coeliac artery were found to stimulate the adrenal glands. The substances mentioned under 4 were tested and were found to have the same actions, except that cocaine did not interfere with stimulation of the adrenal glands.

I wish to express my thanks to Professor J. H. Burn for his advice and guidance throughout this work.

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THE IMPORTANCE OF IONIZATION IN THE ACTIVITY OF SYMPATHOMIMETIC AMINES

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Several examples exist of the importance of ionization to the biological activity of a compound. Albert and his co-workers (Albert, Rubbo, Goldacre, Davey, and Stone, 1945; Albert and Goldacre, 1948) have shown that ionization is one of the limiting factors in the antibacterial activity of the acridines. Bell and Roblin (1942) showed that in the sulphonamide series the presence of both anions and neutral molecules is necessary for activity. Other workers have shown that ionization is disadvantageous to the narcotic activity of the barbiturates (Clowes, Kletch, and Krahl, 1940). Thus it has been established that ionization is an important factor in the activity of synthetic drugs.

The present investigation was designed to investigate the importance of ionization for the activity of naturally occurring physiological agents. The sympathomimetic amines were chosen as a suitable representative series. It was proposed to examine a group of these amines in order to ascertain which particular ionic species was prevalent and also to examine their pharmacological potency at various *p*H values, in order to discover a relation, if any, between pharmacological activity and change of *p*H, i.e. degree of ionization.

Thus it could be ascertained if ionization is an important factor in determining the activity of sympathomimetic amines; which ionic species is responsible for their activity; and whether small changes in *p*H (as might be found in various tissues) would affect their activity either quantitatively or qualitatively.

METHODS

Determination of Dissociation Constants

Potentiometric Method.—In the potentiometric titrations a Stadie electrode system having a glass electrode was used with a Cambridge *p*H meter, and readings were made at 20° C. Pure nitrogen was passed through the apparatus for about 1 hr. before use. The amines were prepared in 0.01M solution and 0.1N-NaOH was added by means of a 2 ml. microburette. To avoid oxidation, particularly of the diphenolic amines, at high *p*H values, the determinations were carried out anaerobically, and all solutions were made up in boiled, glass-distilled water which had been bubbled with nitrogen. The use of 0.1N-NaOH avoided a too-large alteration of volume during titration. A constant ionic strength (0.1) was employed to avoid salt effects, although slight changes occurred during the titrations.

Most of these compounds have two groups which ionize in the *p*H range examined (amino and phenolic), and a third group (secondary phenolic) which ionizes at very high *p*H values. This latter group in catechol was reported by Abichandani and Jatkar (1938) to have a *p*Ka of over 12.0. Thus, the ionization of the primary phenolic group in the diphenols was assumed not to be affected by the presence of the second phenolic hydroxyl.

The *p*Ka's (i.e. negative logarithms of the dissociation constant Ka) were calculated according to the following equation:

$$K_1 = \frac{h\{(c-a) + o\bar{h}\}}{a - o\bar{h}}$$

where K_1 = dissociation constant, h = hydrogen ion concentration, $o\bar{h}$ = hydroxyl ion concentration, a = equivalents of alkali, c = original concentration of base.

Where it was found that the amino and phenolic groups ionize in close proximity to one another on the *p*Ka scale, then in such cases, in order to calculate the *p*Ka values, it was necessary to use the equation of Britton (1942):

$$\frac{a + h - o\bar{h}}{h + 2K_2} = \frac{cK_1}{h^2 + hK_1 + K_1K_2}$$

where K_2 is the second dissociation constant. The *p*H was recorded and the *p*Ka calculated after each of 7 or 8 additions of alkali. Each addition of alkali resulted in approximately 10% neutralization.

Spectrophotometric Method.—The method involved measurement of the optical density of 0.0002M solutions of the amines (adjusted to ionic strength 0.1) at various *p*H's over wavelengths from 260 to 300 mμ. Glycine buffers were used and the optical densities were measured with a Unicam U.V. spectrophotometer. The procedure was carried out in the absence of oxygen. Fig. 1 shows

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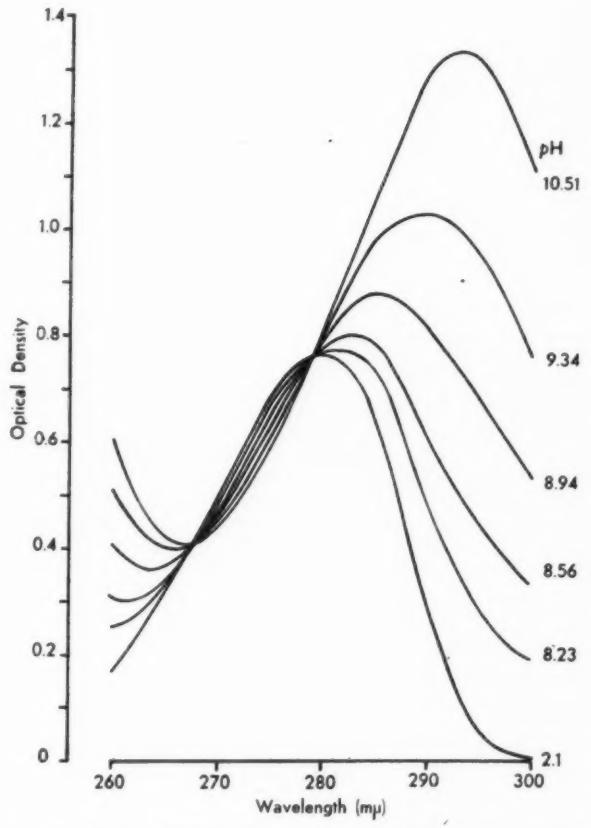


FIG. 1.—To show the change in the ultraviolet absorption spectrum of adrenaline when ionization takes place. This change is shown from unionized molecules at *p*H 2.1 to complete ionization at *p*H 10.51, through four intermediate stages. The concentration of solution was 0.0002M and the ionic concentration 0.1.

typical effects of *p*H on the absorption spectrum of a phenolic amine. The *p*Ka's were obtained from the equation

$$p\mathrm{Ka} = p\mathrm{H} - \log. \left\{ \frac{(\varepsilon - \varepsilon_{AH})}{(\varepsilon_{A^-} - \varepsilon)} \right\}$$

where ε_{AH} and ε_{A^-} are the extinction coefficients of unionized and completely ionized species calculated from the optical densities measured at *p*H 2.1 (ε_{AH}) and *p*H 10.51 (or *p*H 12.48) (ε_{A^-}), ε is the extinction coefficient of the sample at various intermediate *p*H's. Each experiment was carried out and the *p*Ka's calculated at several wavelengths. It was assumed that in this series the presence of the amino group does not affect the absorption spectrum of the phenols.

Pharmacological Methods

The sympathomimetic amines exhibit two types of activity on smooth muscle—relaxation and contraction—according to the preparation employed. In this investigation two preparations were used—isolated rabbit uterus, which is contracted, and isolated rabbit intestine, which is relaxed. Both these tissues were used in a standard organ bath surrounded by a suitable saline (Locke's solution for uterus and Tyrode solution for intestine) maintained at *p*H 7.3 and 37° C., and continuously bubbled with 5% CO_2 in O_2. Each of the amines was assayed against (−)-adrenaline as a standard, and the potency was expressed in terms of adrenaline as 100.

In some experiments the *p*H of the surrounding solution was altered by addition of glycine or glycylglycine buffers. In these experiments the change in the ratio adrenaline activity : amine activity was noted, thus eliminating any tissue reaction to the buffer.

RESULTS

The dissociation constants were determined, where possible, by both methods. It is not possible, using potentiometric titration, to determine which group is ionizing first. Thus, use was made of the spectrophotometer to determine the dissociation of the phenol, as this group has an U.V. absorption spectrum which changes when ionization takes place. There was general agreement between the values obtained by the two methods, those values obtained by the spectrophotometric method being usually 0.1–0.2 of a unit higher. However, with those compounds in which the *p*Ka's of the two ionizing groups were within 0.5 of a unit, there were discrepancies of the order of 0.5. Such compounds were those monophenols containing an alcoholic group in the side chain, e.g. WIN 5512, *p*-sympatol, WIN 833, WIN 5513, and *m*-sympatol. It is considered that when the two *p*Ka's are so close the potentiometric method used here is not sufficiently accurate for direct application of Britton's equation. In these instances the phenolic $p\mathrm{Ka}_1$ was determined by the spectrophotometric method and the basic $p\mathrm{Ka}_2$ calculated from this result and Britton's equation.

The values of the dissociation constants expressed in *p*Ka units are given in Table I. The basic *p*Ka's are greater than 9.4 and the acidic *p*Ka's are, with one exception, over 8.8. Thus, at physiological *p*H, the ionic constitution of these amines is approximately 94% kation, 5% zwitterion, and 1% anion and undissociated molecule. Leffler, Spencer, and Burger (1951) measured the apparent dissociation constants of 27 sympathomimetic amines. Their method was to add to a solution of the salt of each amine the calculated amount of sodium hydroxide solution required for half-neutralization—a procedure which is liable to give rise to undetectable errors. Since the phenolic group dissociates first, these authors were measuring its dissociation in the diphenols, that of the basic group in the non-phenols, and a complex dissociation of the two groups in the monophenols. These results have not been taken into account during the present investigation. The

TABLE I

DISSOCIATION CONSTANTS (± STANDARD ERRORS) OF THE PHENOLIC (pK_1) AND AMINO (pK_2) GROUP OF SYMPATHOMINETIC AMINES

The numbers represent the potency of each amine compared with (−)-adrenaline as 100. In the last column are given the rates of oxidation by amine oxidase relative to that of tyramine as 100.

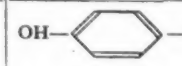
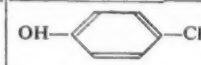
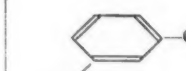
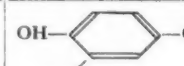
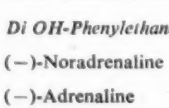
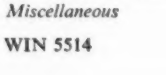
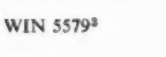
Compound	Substituents on Atoms Indicated by *	pKa_1		pKa_2	Pharmacol. Activity		Enzymatic Oxidation (Blaschko *et al.*, 1937)
		Spectro.	Potentiom.	Potentiom.	Rabbit Intestine	Rabbit Uterus	
Phenylethylamine							
β-Phenylethylamine				9·86 ± 0·06	< 0·01	< 0·01	11
(±)-Phenylisopropylamine				9·93 ± 0·01	< 0·01	0·02	< 2
WIN 5523				10·02 ± 0·06	0·04	< 0·01	—
Phenylethanolamine							
(−)-Ephedrine				9·49 ± 0·05	0·05	0·06	< 2
(−)-Propadrine				9·44 ± 0·04	0·01	0·13	—
Mono OH-Phenylethylamine							
Tyramine		9·77 ± 0·06	9·53 ± 0·03	10·78 ± 0·02	< 0·01[1]	0·05	100
WIN 5565		9·70 ± 0·04	9·49 ± 0·02	10·75 ± 0·04	0·02	< 0·01	—
Mono OH-Phenylethanolamine							
WIN 5512		9·53 ± 0·07		9·70 ± 0·09	0·5	0·05	—
(±)-*p*-Sympatol		9·59 ± 0·05		9·71 ± 0·08	0·5	1·0	48
WIN 833		9·57 ± 0·07		9·78 ± 0·09	1·0	< 0·01	—
WIN 5513		9·56 ± 0·08		9·76 ± 0·09	< 0·01[1]	< 0·01	—
(−)-*m*-Sympatol		9·61 ± 0·09		9·70 ± 0·07	10·0	60·0	59
Di OH-Phenylethylamine							
Hydroxytyramine		8·92 ± 0·06	8·87 ± 0·03	10·63 ± 0·07	0·2	0·2	—
Epinine		8·86 ± 0·08	8·90 ± 0·06	10·61 ± 0·06	8·0	10·0	125

TABLE I—*contd.*

Compound	Substituents on Atoms Indicated by *			pKa_1		pKa_2	Pharmacol. Activity		Enzymatic Oxidation (Blaschko *et al.*, 1937)
				Spectro.	Potentiom.	Potentiom.	Rabbit Intestine	Rabbit Uterus	
Di OH-Phenylethanolamine		—CH	—NH						
(−)-Noradrenaline		H	H	8·90 ± 0·06	8·73 ± 0·04	9·78 ± 0·09	100	90	51
(−)-Adrenaline		H	CH_3	8·88 ± 0·04	8·71 ± 0·06	9·90 ± 0·06	100	100	65
(−)-Isopropylnoradrenaline		H	$CH(CH_3)_2$	8·87 ± 0·07	8·72 ± 0·05	9·87 ± 0·07	130	< 0·01	—
WIN 5589		H		8·89 ± 0·06	8·85 ± 0·04	10·03 ± 0·06	100	0·01	—
(−)-Corbasil		CH_3	H	8·85 ± 0·05	8·75 ± 0·03	9·75 ± 0·09	12	3·3	< 2
WIN 3243		$CH(CH_3)_2$	$CH(CH_3)_2$	8·91 ± 0·08	8·85 ± 0·05	10·00 ± 0·06	5	< 0·01	—
Miscellaneous									
WIN 5514		CH_2	—NH $CH(CH_3)_2$	7·64 ± 0·03	7·53 ± 0·02	9·58 ± 0·09	< 0·01	< 0·01	—
WIN 5505		CH_2	—NH $CH(CH_3)_2$			9·48 ± 0·05	0·5	< 0·01	—
WIN 5503		CH_2	—NH $CH(CH_3)_2$		4·22 ± 0·04[4]	9·60 ± 0·04	0·01	< 0·01	—
WIN 5579[3]		CH_2	—NH $CH(CH_3)_2$			10·23 ± 0·07	< 0·01	< 0·01	—

[1] Caused non-sympathomimetic contraction. [2] Amino group $-N(CH_3)_2$. [3] Phenyl replaced by cyclohexyl. [4] Basic *p*Ka of N atom attached to phenyl ring. — Indicates not tested.

dissociation constants of five of the compounds have been reported by others workers: *laevo*ephedrine 9.48 (Ayrapaa, 1950); β-phenylethylamine 9.85 (Bardinet and Metayer, 1948); tyramine (NH_2) 9.3, (OH) 10.9 (Ogston, 1936); adrenaline (NH_2) 10.2, (OH) 8.7; tyramine (NH_2) 9.3, (OH) 10.9 (Albert, 1952). The first two of these results agree with those found in the present investigation, and those for adrenaline differ only by 0.2–0.3. The results for tyramine, however, are quite different, and it is obvious that the figure given by Ogston (1936) and Albert (1952) for the ionization of the phenolic group has been found in the present investigation to represent that of the amino group and *vice versa*.

Some generalizations may be made on the relationship between chemical structure and dissociation constants in this series of phenylethylamine derivatives. This constant of the basic amino group is reduced by 0.4 of a *p*Ka unit on addition of the alcoholic group when no phenol group is present, and by 0.8 of a unit in mono- and di-phenols. The phenolic phenylethylamines are considerably more basic (0.8 unit) than their non-phenolic analogues. The acidic phenol group is more feebly ionized in the monophenols than in the diphenols. It is interesting to note that, when the alcoholic group in the side chain is replaced by a ketonic group in a *para*-monophenol, the acid is greatly strengthened and the base weakened, e.g. WIN 833, acid 9.59, base 9.78; WIN 5514, acid 7.64, base 9.58. In the cyclohexylethylamine derivative (WIN 5579) the basic group is stronger than that of the corresponding phenylethylamine (WIN 833), while the hydroxyl loses its phenolic character and does not ionize at all.

Table I also shows the relative pharmacological activities of the compounds in relaxing rabbit intestine and in contracting rabbit uterus. It is quite obvious that there is no direct relationship between pharmacological activity and *p*Ka. The only notable feature is that the compounds are present mainly in the kationic form at physiological *p*H. The *p*K's of these compounds are such that to ensure the absence (effectively) of the kationic form, the *p*H would have to be raised to 9.5–10.0, which is unsuitable for a biological tissue. In a series of experiments in which the *p*H of the medium surrounding the biological test tissue was varied, it was found that glycine and glycylglycine buffers (Dernby, 1915) provided a suitable means of raising the *p*H to 8.5–8.7 without any apparent effect on the tissue, but at higher *p*H's irreversible changes took place. There was no change in the relative activities of the compounds over this range of *p*H.

In the last column of Table I are given some figures quoted from Blaschko, Richter, and Schlossman (1937) of relative rates of oxidation by the enzyme amine oxidase. There is no relationship between enzymatic oxidation and *p*Ka, and therefore no evidence regarding the preference of this enzyme for any particular ionic species.

Discussion

The results of this investigation give a general picture of the ionization of the sympathomimetic amines derived from phenylethylamine. The alcoholic group in the side chain has a base-weakening effect whatever other structures are present, while the phenolic hydroxyl strengthens the base. In the presence of a second phenolic group which ionizes only at very high *p*H's, the dissociation of the ionizing phenol is enhanced. Other structural modifications have been made, but these did not result in a considerable weakening of the base. Thus, it seems unlikely from these facts that a sympathomimetic agent based on the structure of phenylethylamine can be derived having a basic *p*Ka of less than 8.0; above this *p*Ka no appreciable change in ionic composition results from small changes in physiological *p*H.

It is concluded that the main question—how important ionization is for the activity of sympathomimetic amines—cannot be answered. It can be stated, however, that ionization cannot account for the differences in activity among sympathomimetic amines derived from phenylethylamine. Further, at physiological *p*H the kationic form of these compounds is by far in excess of any other ionic species and is thus probably responsible for pharmacological activity, but other possibilities cannot be excluded.

Summary

1. A series of sympathomimetic amines has been examined in order to discover the importance of ionization for their pharmacological activity.

2. These amines are strong bases of *p*Ka (amino) 9.4–10.8, and weak acids of *p*Ka (phenolic) 8.8–9.5.

3. Ionization is not important in accounting for the differences in activity of these amines.

4. At physiological *p*H the compounds are present almost wholly as kations.

It is a pleasure to acknowledge much helpful discussion with Dr. A. Neuberger of this Institute.

All the compounds designated with WIN numbers were kindly supplied by Dr. M. L. Tainter, Director of the Sterling-Winthrop Research Institute, New York.

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QUANTITATIVE DETERMINATION OF MAST CELL FRAGMENTATION BY COMPOUND 48/80

BY

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(RECEIVED AUGUST 4, 1954)

Compound 48/80 as reported by Baltzly, Buck, de Beer, and Webb (1949) is a polymer of *N*-methylhomoanisylamine and formaldehyde. Although similar LD 50's have been obtained with different batches of Compound 48/80 (Dews, Wnuck, Fanelli, Light, Tornaben, Norton, Ellis, and de Beer, 1953), it was felt desirable to have an assay method which might be more closely related to the histamine-liberating property of 48/80.

Compound 48/80 was described as a histamine liberator by Feldberg and Paton (1951) and Paton (1951). Recently, Mota, Beraldo and Junqueira, (1953) and Fawcett (1954) have shown the *in vivo* fragmentation of mast cells by 48/80. A relatively simple technique for *in vitro* fragmentation of the mast cells in pieces of rat mesentery was also used by Mota. I felt this latter method could be adapted to quantitative determination of mast cell fragmentation. The method described here is based on the observation that at low concentrations of 48/80 the proportion of cells fragmented depends on the concentration of 48/80.

METHODS

Adult male albino CF1 rats were killed with ether. Pieces of mesentery with connecting lobes of fat and blood vessels were rapidly dissected out and placed in Ringer-Locke solution (NaCl 0.9%, KCl 0.042%, $CaCl_2$ 0.024%, $NaHCO_3$ 0.015%, and dextrose 0.1%). From 10 to 15 pieces were obtained from each rat. This procedure required about 5 min. The pieces were then placed in solutions of 48/80 in Ringer-Locke for 30 ± 1 min. The pieces of mesentery were removed to a 4% formaldehyde solution containing 0.1% toluidine blue for 20 to 30 min. and then transferred through acetone (two changes) and xylene (two changes) and mounted on slides. Before mounting, excess pieces of fat were trimmed from the edges of the mesentery.

All solutions were at room temperature. Usually two pieces of mesentery were used in each concentration of drug. From these pieces five microscope fields were selected at random under 100 × magnification from widely separated areas of the mesentery. In each field, using 430 × magnification, the first 10 mast cells were examined, starting from the left-hand side of the field and proceeding clockwise. For each drug concentration 50 cells were counted and the percentage of disrupted cells was obtained. Each cell was considered either "disrupted" or "not disrupted." The term "disrupted" was selected instead of "fragmented," since granules were found around many cells which did not appear to be in fragments. Fawcett (1954) has also reported this phenomenon. The sole criterion for calling a cell "disrupted" was the presence of granules outside the cell. Many cells which did not show extrusion of granules appeared swollen at low concentrations of 48/80.

For evaluating the percentage disruption the labels of the slides were covered and the slides were counted in random sequence.

RESULTS

Comparison of Different Batches of 48/80.—Dose-response curves for the percentage disruption of mast cells were obtained for different batches of 48/80 with 0.2, 0.4, and either 0.1 or 0.8 μg./ml. of 48/80. Control mesentery with Ringer-Locke solution alone was run with each experiment. Seven batches of 48/80 were tested. The percentage disruption at each concentration is given in Table I.

TABLE I

DISRUPTION OF MAST CELLS BY DIFFERENT BATCHES OF 48/80

Batch No.	Expt. No.	Percentage Disruption					ED 50 (μg./ml.)	Fiducial Limits (P=0·95)
		Concn. of 48/80 (μg./ml.)						
		0	0·1	0·2	0·4	0·8		
NS 280	1	10		28	76	92	0·27	·23–·32
	2	6		26	68	90	0·30	·26–·35
	3	8		54	66	86	0·20	·12–·31
	4	2	28	68	76		0·16	·12–·19
	5	6	12	48	70		0·22	·19–·26
AE 92	3			56	82	86	0·13	·07–·26
AE 2	1			40	88	100	0·23	·20–·26
	2			4	68	100	0·33	·28–·39
GD 78	3			40	64	72	0·26	·19–·35
VI 64	1			44	54	62	0·30	·16–·56
VI 53	3			6	28	90	0·47	·42–·53
VI 65	3			12	36	64	0·57	·45–·70

The ED 50 values (50% disruption of mast cells) were calculated by probit analysis, following the method of Bliss as described by Goulden (1952). The calculated ED 50's and fiducial limits are given in the last two columns of Table I. Batch NS 280 was selected as the standard. The ED 50's for two batches (VI 53 and VI 65) were outside the range found for NS 280.

Mechanism of Disruption of Mast Cells.—Mota *et al.* (1953) observed the disruption of mast cells in 48/80 solutions using phase-contrast microscopy, and described the process as "bubbling." In mast cells vitally stained with neutral red I have observed a similar phenomenon caused by 48/80. When a piece of mesentery is placed in a solution of 48/80 the cell swells and becomes balloon-like, sometimes twisting and turning as it swells, possibly in response to the mechanical forces of the surrounding tissue.

Since living cells swell before they fragment, and since stained intact cells appear swollen, it was assumed that at least one of the effects of 48/80 was to increase the osmotic pressure within the mast cell and hence cause an inflow of water. This increase in osmotic pressure might come about in several ways:

(1) The mast cells might preferentially concentrate 48/80.

(2) A substance which was previously not available for osmotic effect might be liberated or formed in the cell.

(3) The cell might take up or become more permeable to ions present in the extracellular fluid.

An experiment was designed to obtain evidence regarding these possibilities. If the third mechanism listed above is correct, it would follow that diluting the extracellular fluid and reducing the concentrations of extracellular ions would decrease the effectiveness of 48/80. Such a procedure would tend also to cause mast cells to swell in hypotonic solutions. The effect of 48/80 would, however, be expected to be enhanced by hypotonic solutions if the first or second possibility were true.

Table II gives the results of two experiments in which concentrations of 0.05, 0.1, 0.2 and 0.4 μg./ml. of 48/80 were used in combination with undiluted Ringer-Locke, and with Ringer-Locke diluted by 1/4 and 1/2 with distilled water.

In Fig. 1 the percentage of disruption observed in the controls was subtracted from each reading for that experiment. The values for the two experiments were averaged.

TABLE II

PERCENTAGE DISRUPTION OF MAST CELLS BY 48/80 IN ISOTONIC AND HYPOTONIC SOLUTIONS

Concn. of 48/80 (μg./ml.)	Expt.	Dilution of Ringer-Locke		
		Undiluted	1 Part Water, 3 Parts Ringer-Locke	1 Part Water, 1 Part Ringer-Locke
0	1	6	8	48
	2	2	28	66
0·05	1	6	2	88
	2	8	18	58
0·1	1	12	14	68
	2	28	38	98
0·2	1	48	26	54
	2	68	32	82
0·4	1	70	22	38
	2	76	54	60

From the data in Table II and the curves in Fig. 1, it appears that dilution of Ringer-Locke solution inhibits the disruption of mast cells by

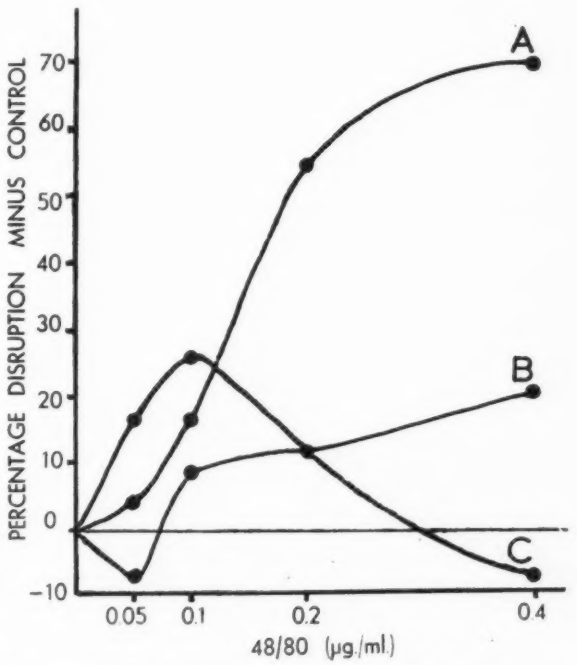

FIG. 1.—Effect of diluting Ringer-Locke solution on disruption of mast cells by 48/80. A = undiluted. B = 3 parts Ringer-Locke, 1 part distilled H_2O. C = 1 part Ringer-Locke, 1 part distilled H_2O.

48/80. The evidence, therefore, is in favour of the possibility that 48/80 may make the cell more permeable to extracellular ions and thereby cause disruption of the cell. The evidence is, of course, indirect and no explanation is advanced for the particular shape of the curves in hypotonic solutions. The shapes of these curves were, however, fairly similar in both experiments.

Rate of Action of 48/80 on Mast Cells.—If pieces of mesentery are allowed to remain in Ringer-Locke solution the number of cells spontaneously releasing granules increases with time. Table III gives the results of an experiment varying the time

TABLE III

PERCENTAGE DISRUPTION OF MAST CELLS AFTER VARIOUS LENGTHS OF EXPOSURE TO 48/80

Concn. of 48/80 (μg./ml.)	Time of Exposure (Min.)			
	1	15	30	60
0	—	0	2	—
	—	—	8	4
0·05	—	8	8	—
0·1	—	36	28	—
0·2	0	50	68	—
	—	—	54	54
0·4	2	—	66	82
	40	44	76	90
0·8	16	—	86	98

of exposure to 48/80. At a concentration of 0.2 μg./ml. disruption increased markedly during the first 15 min. of exposure to 48/80. After this there is little increase in the number of disrupted cells. At higher concentrations (0.4 and 0.8 μg./ml.), the rate of disruption is rapid up to 30 min. and decreases between 30 and 60 min. These changes in rate are shown in Fig. 2.

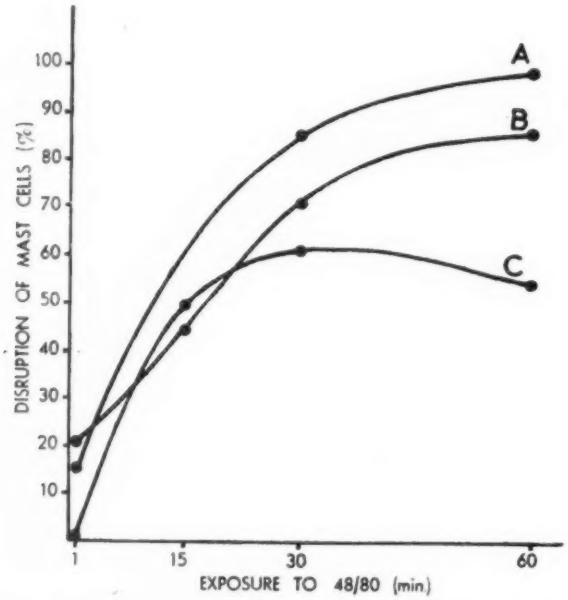

FIG. 2.—Rate of action of 48/80 on mast cells. A=0·8 μg. 48/80/ml. B=0·4 μg. 48/80/ml. C=0·2 μg. 48/80/ml.

A standard immersion period of 30 min. was, therefore, chosen for these experiments.

DISCUSSION

Using the method described here for measuring the effect of compounds on mast cells, sigmoid dose-response curves are obtained when the concentration of 48/80 in Ringer-Locke is plotted against the percentage disruption (Fig. 1). An ED 50 can be calculated for such curves. With this method, it is possible to compare quantitatively the effectiveness of compounds which disrupt mast cells. This method also makes it possible to obtain quantitative evaluations of compounds which block this action.

The error of the ED 50's, in the experiments with the best distribution of data in Table I, is somewhat less than 20%. Presumably the error of the method could be decreased by increasing the number of points establishing the dose-response curves.

Although 30 min. was selected as the time allowed for the action of solutions of 48/80 it is possible that somewhat less error would have been obtained if the time had been increased to 60 min. This would appear to be closer to the time required for the maximum effect of 48/80 at concentrations above 0.2 μg./ml. The increased time needed with higher concentrations of 48/80 may be dependent on the time needed to achieve equilibrium, since higher concentrations would take longer to reach equilibrium under conditions such as exist with passage across a semi-permeable membrane.

The dissimilarity of the shapes of the curves in Fig. 1 may be postulated to reflect two processes acting to disrupt the mast cells. One process, previously discussed, would be that 48/80 makes the cells more permeable to extracellular ions. This process would be responsible for all of the disruption produced by concentration of 48/80 above 0.1 μg./ml. However, at concentrations of 0.1 and 0.5 μg./ml., another process might be operating to produce some disruption. This would account for the fragmentation observed in these low concentrations of 48/80 in diluted Ringer-Locke. Such a process could be either of the possibilities mentioned previously as alternatives to the effect of 48/80 on permeability to extracellular ions. Further studies are in progress, to obtain more data regarding these hypotheses.

SUMMARY

1. A quantitative method for evaluation or disruption of mast cells by compound 48/80 is described. With this method, ED 50 values can be calculated by probit analysis and used to determine differences in various batches of 48/80.

2. Preliminary experiments to study the method by which 48/80 disrupts mast cells support the hypothesis that, in solutions of 48/80, the mast cells become more permeable to extracellular ions.

3. The time required for maximum effect of solutions of 48/80 on mast cells varies between 30 and 60 min., depending on the concentration of 48/80.

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IDENTIFICATION OF AN ISOPRENALINE-LIKE SUBSTANCE IN EXTRACTS OF ADRENAL GLANDS

BY

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(RECEIVED AUGUST 16, 1954)

A sympathomimetic amine, not hitherto described as naturally occurring, was unexpectedly encountered in an experiment in which the adrenaline and noradrenaline, in saline extracts of cat adrenal glands, were being separated chromatographically.

In this experiment a chromatogram, made from an aliquot of extract corresponding to just less than one-third of a cat gland, was oxidized, by spraying with a solution of potassium ferricyanide, to convert any primary or secondary catechol amines to the corresponding adrenochromes. Adrenaline and noradrenaline then appeared as red and pink spots respectively, and were identified by means of their R_F values. There was, however, an additional very pale pink spot (third amine), with an R_F value of approximately 0.7, lying ahead of the adrenaline area from which it was separated by a negative horizontal band of paper. Corresponding bands from another, similar, and adjacent chromatogram were therefore eluted and prepared for biological testing. The eluate from the negative zone separating the adrenaline area from that of the third amine was pharmacologically inert, whereas that from the zone of the third amine was highly active. Moreover, the type of activity encountered in the latter zone differed markedly from that obtained from the adrenaline and noradrenaline areas. The third amine, like adrenaline and unlike noradrenaline, strongly antagonized the action of acetylcholine on the rat uterus. However, when doses of adrenaline and of third amine eluates, which had proved equally active on the rat uterus, were injected intravenously into a cat under chloralose, the adrenaline eluate raised the mean arterial pressure and caused contraction of the nictitating membrane as expected, but the eluate of third amine lowered the blood pressure, caused tachycardia, and was without action on the nictitating membrane.

The results of a further investigation of this third amine are presented here in three sections. The first section is concerned with the great similarity between the third amine and isoprenaline. The second section deals with the presence or absence of the third amine in the adrenal glands of various species. Finally, the properties and actions of the third amine have been contrasted with those of lactyladrenaline and lactylnoradrenaline, because these two compounds are known sometimes to arise as artifacts in extracts of adrenal glands. Lactyladrenaline was first isolated by Kendall (1932) and was studied, together with lactylnoradrenaline, by Crawford (1951). Work by Serlin and Goldenburg (1953) on the instability of synthetic noradrenaline in acid ethanol indicated that these so-called lactyl derivatives may prove to be ethers. Their hypothesis has not been investigated; the terms lactyladrenaline and lactylnoradrenaline are here used to refer to compounds which are believed identical with those previously encountered in extracts of adrenal glands by Crawford.

METHODS

Collection of Adrenal Glands.—Adrenal glands were removed from spinal cats or from cats under anaesthesia (ether, chloroform, chloralose, or pentobarbitone), and from rabbits and guinea-pigs immediately after death from a blow on the head. Cat, rabbit, and guinea-pig glands were at once put into a deep freeze at −20° C. Human and monkey glands were removed under ether anaesthesia, and were quickly carried to the laboratory in dry tubes surrounded with ice in a thermos flask.

Preparation of Solutions for Chromatography.—The procedure described below is that finally adopted and used for glands of all species. Frozen glands were rapidly weighed, cut into small pieces, and ground in ice-cold saline (0.9% NaCl, 2 ml./gland) within 2 hr. of excision from the animal. Coarse debris was removed from the saline extract by centrifuging at 3,000 rev./min. for 3 min. Usually 0.3 ml. M/15 phosphate buffer, *p*H 7.4, and 0.2 ml. 0.9% NaCl were added to each 0.5 ml. aliquot of supernatant fluid; sometimes the buffer was replaced by additional saline. Proteins were then precipitated by the addition of 7 ml. ethanol and 0.25 ml. 4N-HCl.

After centrifuging, 7.5 ml. of supernatant fluid was evaporated to dryness *in vacuo* at a temperature not exceeding 50° C. The residue was extracted with 3 ml. of a mixture of equal parts of acetone and ethanol; 2.5 ml. of this acetone-alcohol extract was evaporated to small volume and used for chromatography.

Chromatography and Elution.—The methods used for chromatography, for the development of chromatograms, for elution, and for the preparation of eluates for biological testing have been described in full detail elsewhere (Vogt, 1952) and need only be summarized here. The concentrated acetone-alcohol extracts were quantitatively transferred to acid-washed Whatman No. 1 filter paper. The area of the paper to be traversed by gland samples destined for use in bioassays had previously been sprayed with a solution of ascorbic acid 50 mg./100 ml. Phenol containing 15% w/v 0.1 N-HCl was used as solvent, and ascending chromatograms were run in an atmosphere of CO_2. Phenol was removed by washing with benzene, and the whole process, including drying, was carried out at room temperature. R_F values were determined from chromatograms developed by spraying with 0.44 g. potassium ferricyanide dissolved in 100 ml. 0.2 M-phosphate buffer, *p*H 7.8.

Adrenaline, noradrenaline, isoprenaline, and lactyladrenaline were used as reference compounds, Chromatograms of not less than two samples from a single gland, and of mixed reference compounds, were made in parallel on a single sheet of paper. The solvent front was allowed to advance 14–16 cm. beyond the line of application of the extracts before the process was interrupted. Chromatograms from one sample of each gland, and of the reference compounds, were used for determination of R_F values; those of other gland samples were eluted and prepared for bioassay as described by Vogt (1952).

Pharmacological Methods.—Adrenaline and noradrenaline were assayed either on the blood pressure and nictitating membrane of a spinal cat, or on the rat uterus and colon as described by Gaddum, Peart, and Vogt (1949). The third amine was compared with isoprenaline in parallel quantitative assays in which at least three—and usually four, and sometimes five—different tissues drawn from two different species were employed. The pharmacological differentiation of the new amine from adrenaline was greatly facilitated by the use of an atypical strain of rats obtained, accidentally, from Taylor (London). The uteri, colons, and ileums of these rats were remarkably insensitive to adrenaline, but were normally sensitive to isoprenaline and to the third amine (Table I). Assays of the third amine on the blood pressure, nictitating membrane, and bronchiolar resistance of cats were carried out under chloralose anaesthesia.

Drugs.—(−)-Adrenaline tartrate (Burroughs Wellcome, Ltd.), (−)-noradrenaline and (±)-isoprenaline (Sterling Winthrop) were obtained commercially. Lactyladrenaline and lactylnoradrenaline were synthesized as described by Crawford (1951).

TABLE I

THE RELATIVE SENSITIVITY OF TISSUES FROM DIFFERENT STRAINS OF RATS TO (−)-ADRENALINE AND (±)-ISOPRENALINE

Rats		Ratio of Equally Effective Doses, (−)-Adrenaline/(±)-Isoprenaline, Expressed as a Range			
Source	No. Examined	Ileum	Colon	Uterus	
Gaddum, Peart and Vogt (1949) ..	—	—	1	1–2	(typical)
Chester Beatty Institute	4	1–50	1–3	1–2	(typical)
London School of Pharmacy	8	1–11	1–3·5	1–3	(typical)
Burroughs Wellcome Ltd.	4	1–5	1–2	1–3	(typical)
Old assay rats and Taylor (London) ..	25	35–65	20–60	12–50	(atypical)

RESULTS

The Properties of the Third Amine

Pharmacological.—Preliminary experiments had shown that the pharmacological properties of the third amine differed from those of adrenaline and noradrenaline, and resembled those of isoprenaline. Both the third amine and isoprenaline caused a fall

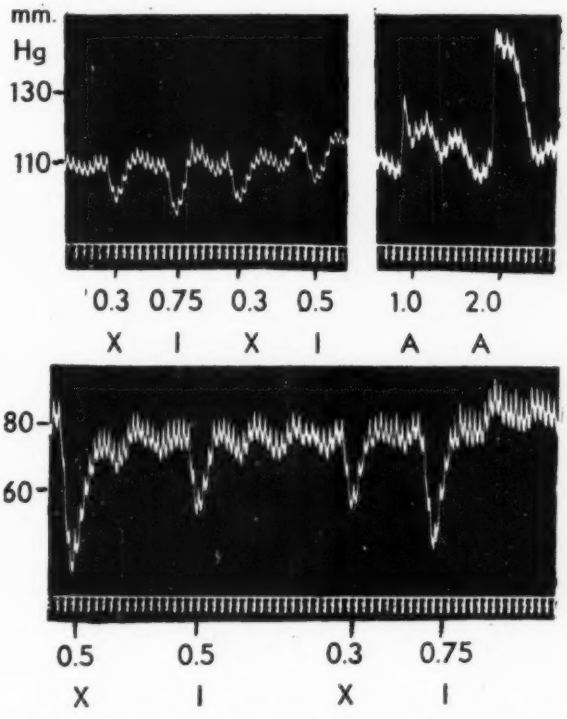

FIG. 1.—Potentiation of the depressor actions of isoprenaline and third amine by tolazoline. Cat, under chloralose. Arterial blood pressure before (upper record) and after (lower record) 7 mg. tolazoline HCl. All drugs injected i.v. A, (−)-adrenaline, in μg.; I, (±)-isoprenaline in μg.; X, an eluate of third amine, from cat adrenal, in ml. Time, 10 sec.

in blood pressure and tachycardia when injected intravenously into spinal cats or cats anaesthetized with chloralose, and their depressor actions were equally potentiated by adrenaline antagonists (Fig. 1). This indicated that both amines have a weak but comparable vasoconstrictor action which is normally masked by their stronger dilator effect, and is abolished by sympatholytic drugs. The effect of third amine on the nictitating membrane varied from cat to cat, but always in parallel with that of isoprenaline. Isoprenaline, 0.5 to 2 μg., was without effect on the nictitating membrane of 10 animals, caused contraction in 7, and relaxation in 3. Cocaine, injected subcutaneously into cats in doses of 5 to 7 mg./kg., affected the responses of the blood pressure and nictitating membranes to the two amines similarly. An initial apparent potentiation of their depressor action was only once observed, and was followed by the typical change seen in each of three other experiments: the depressor actions of these two amines were converted to pressor activities, and their power to cause contraction of the nictitating membrane was potentiated, in parallel (Fig. 4). Further remarkable similarity between the two amines became apparent after the subcutaneous injection of pilocarpine nitrate, 0.5 mg./kg., into cats. Pilocarpine caused great and selective potentiation of the contractions of the nictitating membrane in response to isoprenaline and the third amine (but not in response to adrenaline and noradrenaline), and converted their depressor to pressor activity. Moreover, doses of these two amines, which were equi-effective on the blood pressure and on the nictitating membrane, were equally powerful in the relief of pilocarpine-induced bronchospasm (Fig. 2).

Isoprenaline and the third amine were so alike pharmacologically that they could not be distinguished from one another by means of parallel quantitative assays. Fig. 3 illustrates one such assay, in which an eluate of third amine obtained from cat adrenal gland was compared with isoprenaline. The top records in Fig. 3 show that 1 ml. of this eluate was about equal to 1 μg. (±)-isoprenaline in causing contraction of the nictitating membrane and vasodepression in a cat under chloralose. The centre tracing shows that 1 ml. of the eluate equated with 1 μg. (±)-isoprenaline as an antagonist of acetylcholine on the rat colon. The lowest record demonstrates that 1 ml. of the eluate had more than the activity of 0.7 μg., and less than that of 1.3 μg., (±)-isoprenaline in inhibiting the action of acetylcholine on the rat uterus. Since the ratios of the equiactive doses, (−)-adrenaline/(±)-isoprenaline, were, for these particular tissues, uterus 40–45 and colon 1.0 (see Methods) the activity attributed to the third amine cannot have been due to some adrenaline which had become displaced by an overloading of the paper during chromatography. Fig. 4 shows another comparison, this time between isoprenaline and an eluate of third amine obtained from human adrenal gland. The top records are

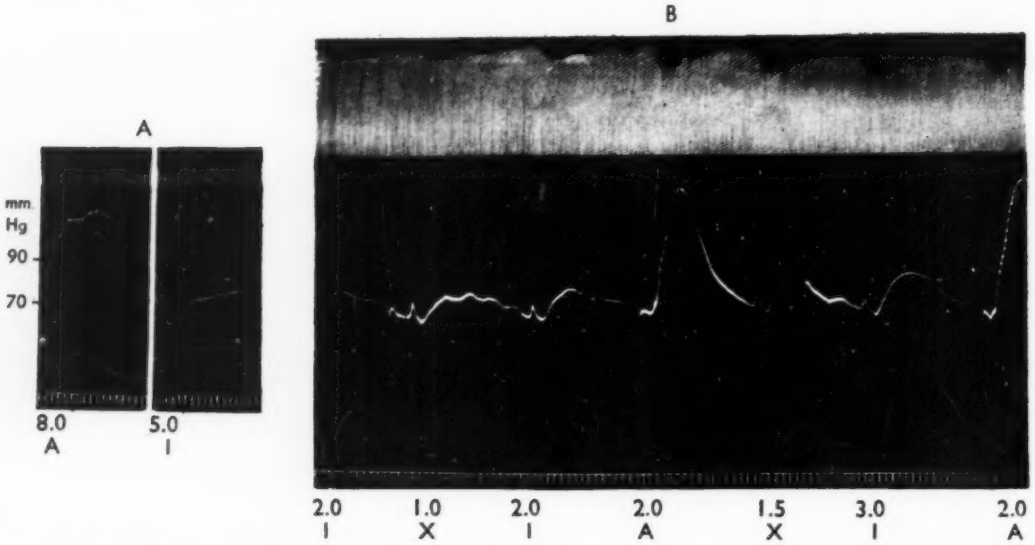

FIG. 2.—Comparison of third amine with isoprenaline, before and after pilocarpine. Cat, under chloralose. Records, from above, are of bronchiolar resistance, arterial blood pressure, and contractions of nictitating membrane —A, before, and B, after, 0.85 mg. pilocarpine nitrate s.c. Other drugs injected i.v. in the doses indicated. A, (−)-adrenaline in μg.; I, (±)-isoprenaline in μg.; X, eluate of third amine, from monkey adrenal, in ml.

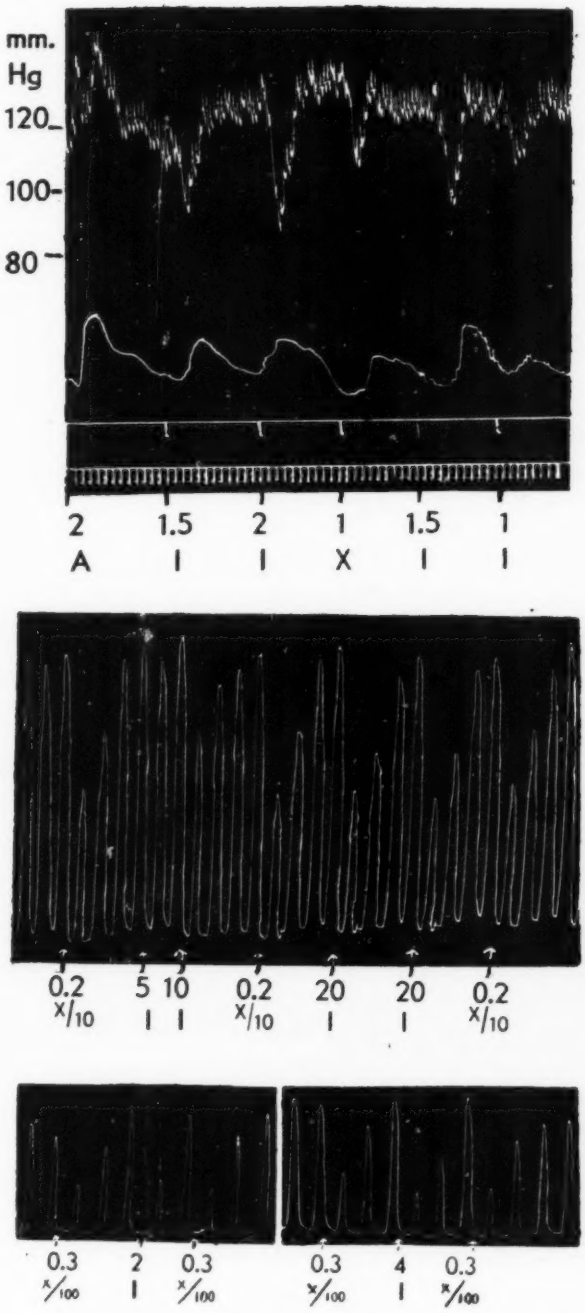

FIG. 3.—Comparison of third amine, from cat adrenal, with isoprenaline, in parallel assays. *Top record*, from cat under chloralose, shows, from above, arterial blood pressure, nictitating membrane, signal, and time in 10 sec. Drugs were injected i.v. Doses of I and of A in μg., and of X in ml. *Middle record*, isolated rat colon responding to 0.1 μg. acetylcholine added at min. intervals, and in contact for 15 sec. At each arrow an inhibitory drug was added 30 sec. before the next addition of acetylcholine. Doses of I in ng., of X in ml. *Bottom record*, rat uterus, contracting every 3 min. in response to 3 μg. acetylcholine acting for 45 sec. Arrows mark additions of inhibitory drugs, 60 sec. before the next addition of acetylcholine. Doses of I in ng., of X in ml. *In all records*, A is (−)-adrenaline, I is (±)-isoprenaline and X is eluate of third amine.

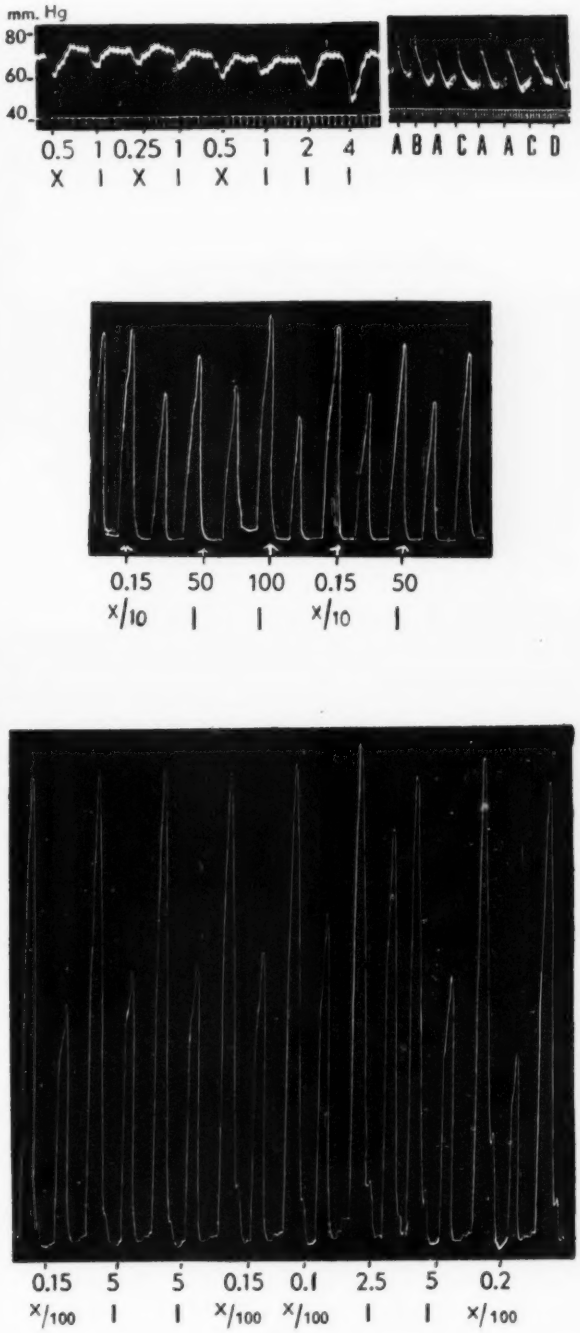

FIG. 4.—Comparison of third amine, from human adrenal, with (±)-isoprenaline, in parallel assays. *Top record*, arterial blood pressure of spinal cat. Drum slowed, and 12 mg. cocaine HCl given s.c., between the 2 parts. Other drugs injected i.v. A, 1.5 μg.; B, 2.5 μg., and D, 1.0 μg., isoprenaline; C, 0.5 ml. eluate of third amine. In *middle record* (rat colon) and *bottom record* (rat uterus) the contractions are in response to a fixed dose of acetylcholine; inhibitory drugs were added as indicated. I, (±)-isoprenaline in μg. in *top record* and in ng. in others; X, eluate of third amine in ml.

of mean arterial pressure, and were taken from a spinal cat weighing 1.9 kg. The first tracing shows that 0.5 ml. of the eluate had more depressor action than 1 μg. and less than that of 2 μg. (±)-isoprenaline. After the subcutaneous injection of 12 mg. cocaine hydrochloride, 0.5 ml. of the eluate had a pressor activity approximately equal to that of 1.5 μg. (±)-isoprenaline. The two lower records show that 0.5 ml. of the eluate equated with 1.7 μg. of (±)-isoprenaline as an antagonist of acetylcholine, both on the rat colon (centre) and on the rat uterus (lowest). The ratios of equally effective doses, (−)-adrenaline/(±)-isoprenaline, were, for these particular rat tissues, uterus 2.0 and colon 56.

The great similarity between isoprenaline and the third amine was not confined to pharmacological activity.

Colour Reactions.—Both third amine and isoprenaline gave green colours with ferric chloride and very strong adrenochrome reactions when treated with oxidizing reagents, and fluorescence was obtained in the ultraviolet for both compounds after treatment with ferricyanide. Adrenochrome formation is typical of catechol amines with primary and secondary amino nitrogen in the side chain, and fluorescence could not occur were the hydroxyl group in the side chain substituted (Lund, 1949).

R_F *Values.*—So great was the similarity between the R_F values obtained for isoprenaline and for the third amine that isoprenaline has been used successfully throughout this work as a chromatographic indicator for the position of the third amine.

Table II summarizes the R_F values which were obtained simultaneously for synthetic compounds and for gland amines. There was no significant difference between the R_F values for natural and synthetic adrenaline and noradrenaline, or between the values obtained for isoprenaline and the third amine.

The Incidence of the Third Amine in the Adrenal Glands of Various Species

The Criteria Adopted for the Identification of the Third Amine.—The existence of the third amine in an extract of adrenal gland was not considered to have been proved until each of three conditions had been satisfied. These criteria were: (i) An R_F value and (ii) a depressor action similar to those of isoprenaline; (iii) an inability to distinguish between the amine and isoprenaline by means of parallel quantitative assays carried out on at least three tissues drawn from two different species. In addition, the tissues selected included either a rat uterus or a rat colon which was abnormally insensitive to adrenaline, but which had normal sensitivity to isoprenaline (see Table I and colon, Fig. 5). The latter precaution was adopted so that no possibility of confusion between adrenaline and the third amine should exist.

The Frequency with which the Third Amine has been Encountered in the Adrenal Glands of Various Species can be read from Table II. This shows R_F values, simultaneously determined, for reference compounds and for gland amines. When a gland chromatogram showed no visible spot for an amine, the R_F value for the corresponding reference compound in that particular experiment was omitted in the compilation of the Table. Since adrenaline was present in every gland chromatogram, the total number of animals in each species that was investigated may be found in brackets following the mean R_F value ± the standard error for gland adrenaline. The number of animals of that species in which third amine was found follows the mean R_F value ± S.E. for third amine in brackets. It was found in the gland extracts from all 4 monkeys, from all 3 human beings, and from three-quarters of the cat population examined. It was occasionally encountered in extracts from rabbit glands, but never in those from guinea-pig glands. Third amine is also

TABLE II

SIMULTANEOUS DETERMINATIONS OF THE R_F VALUES OF SYNTHETIC AMINES AND THOSE OCCURRING IN SALINE EXTRACTS OF ADRENAL GLANDS

(Solvent, phenol containing 15% w/v 0·1N-HCl. For explanation of numerals in parentheses, see text.)

Source	Noradrenaline	Adrenaline	Isoprenaline	Lactyladrenaline
Synthetic	0·210 ± 0·010 (19)	0·486 ± 0·009 (28)	0·684 ± 0·008 (17)	0·801 ± 0·021 (5)
			Third amine	
Cat	0·207 ± 0·007 (12)	0·463 ± 0·019 (12)	0·699 ± 0·026 (9)	0·780 ± 0·051 (2)*†
Monkey	0·203 ± 0·037 (4)	0·459 ± 0·044 (4)	0·672 ± 0·029 (4)	0·820 (1)†
Rabbit	0·210 ± 0·010 (2)	0·504 ± 0·018 (5)	0·675 ± 0·122 (2)	—
Guinea-pig	0·230 ± 0·001 (2)	0·496 ± 0·009 (6)	—	0·780 ± 0·017 (2)**
Man	—	0·490 ± 0·010 (3)	0·707 ± 0·118 (3)	

* Gland stored in deep freeze. † Extract stored overnight in refrigerator after precipitation of proteins with ethanol and acid.

apparently absent from ox adrenal gland. By courtesy of Messrs. Allen and Hanburys Ltd. residues obtained after extraction of the corticoids, and equivalent to a ton of fresh ox adrenal gland, were worked up by Mr. Eastland to an ammoniacal concentrate of a total volume of 2 l. This concentrate yielded 127 g. of crude adrenaline, but neither the crude adrenaline nor the mother liquors contained demonstrable third amine.

The Quantity of Third Amine Present in Extracts of Adrenal Glands.—Only very small amounts of third amine have been encountered. It has been measured as (±)-isoprenaline÷2, and is entered in column 5 of Table III as a percentage of the total amine extracted from the glands of the various species. The second column of this table shows the total weight of amine obtained per gram of fresh gland; the yields were low, indicating that only a part of the medullary amine went into solution during mild extraction with ice-cold saline. The very low values obtained for human gland are probably explained by the fact that two of the three glands were cancerous, and all of them had been cut open and scraped during examination by the surgeons. Glands from other species were always obtained in intact form. The last three columns of Table III record the relative amounts of (−)-adrenaline, (−)-noradrenaline, and third amine found in extracts of the adrenal glands of the different species, expressed as a mean % of the total amine ± the standard error of the mean.

TABLE III

THE PERCENTAGE DISTRIBUTION OF SYMPATHOMIMETIC AMINES IN SALINE EXTRACTS OF THE ADRENAL GLANDS OF VARIOUS SPECIES

Third amine measured as (±)-isoprenaline÷2. The values in the Table are the means ± their standard errors.

Species, and No. Used	Mg. Amine Extracted from 1 g. Fresh Whole Gland	% Total Extracted Amine		
		(−)-Adrenaline	(−)-Noradrenaline	Third Amine
Cat (12) ..	0·967 ± 0·135	74·37 ± 2·89	23·42 ± 3·23	2·21 ± 0·51
Monkey (4)	1·062 ± 0·350	94·93 ± 0·80	3·72 ± 0·96	1·35 ± 0·43
Man (3) ..	0·123 ± 0·241	86·33 ± 1·34	13·30 ± 0·82	0·53 ± 0·24
Rabbit (5)	0·530 ± 0·111	95·32 ± 0·47	4·44 ± 0·24	0·24 ± 0·42
Guinea-pig (6)* ..	0·351 ± 0·098	93·87 ± 3·32	6·13 ± 3·37	Nil

* Groups of animals.

Very small quantities of noradrenaline, which had not been visible on the developed chromatograms, were frequently found on elution of the appropriate areas of parallel chromatograms from human, guinea-pig, and rabbit glands. This noradrenaline was estimated either on the rat colon, or on the blood pressure of a spinal cat, and the identity of the amine was always confirmed by demonstration of a typically weak action as an antagonist of acetylcholine on the rat uterus. Third amine, like isoprenaline, gave a much stronger adrenochrome reaction than noradrenaline, and was therefore more readily visible than the latter amine when present in trace quantities.

The Properties of Lactyladrenaline and Noradrenaline Contrasted with those of the Third Amine

Both lactyladrenaline and lactylnoradrenaline were found in extracts made from a human phaeochromocytoma, which had been cut in pieces and stored in 0.1N-HCl by Crawford in 1951. Crawford obtained R_F values in phenol of 0.62 (lactylnoradrenaline) and 0.75 (lactyladrenaline) both for his synthetic and naturally occurring compounds.

R_F *Values.*—In my experiments the R_F values for synthetic lactyladrenaline and lactylnoradrenaline in phenol have ranged from 0.74–0.86 and 0.55–0.58 respectively, and confirm Crawford's findings. Lactylnoradrenaline was not further examined, because the R_F values obtained in phenol were too low to permit of its identification with the third amine. Lactyladrenaline did, however, require full investigation. The R_F values obtained for synthetic lactyladrenaline have been consistently higher than those for the third amine (Table II), and lactyladrenaline has only been encountered as an artifact either when the glands were stored overnight in the deep freeze, or when extracts from which the proteins had been precipitated with acid alcohol had been stored in the refrigerator. On one occasion both lactyladrenaline and third amine were present in a chromatogram from a stored monkey gland.

Moreover, when an aqueous eluate of chromatographically pure lactyladrenaline (R_F value in phenol 0.79) was re-chromatographed, two spots appeared, one of R_F value 0.80, and another which corresponded in R_F value to the adrenaline reference spot. By contrast, re-chromatography of an eluate of third amine yielded one spot only which had an R_F value identical with that of the original substance.

Colour Reactions.—Whereas both lactyladrenaline and the third amine gave typical adrenochrome reactions, that from the third amine fluoresced in ultraviolet light, but that from lactyladrenaline did not. Moreover, when chromatograms of the two compounds were sprayed first with alkaline hydroxylamine solution and then with acid ferric chloride (Whittaker and Wijesundera, 1952) only with lactyladrenaline

was there any fugitive purple colour amongst the general medley of colours.

Pharmacological Actions.—Lactyladrenaline had only very weak pharmacological actions which resembled those of adrenaline, and which may have been in large part due to partial hydrolysis, of the ester link in aqueous solution (see above). Fig. 5 illustrates the similarity between the effects of adrenaline and of a solution of chromatographically pure lactyladrenaline 500 μg./ml. The top set of records show that 10 μg. (−)-adrenaline and 0.15 ml. of the latter solution caused similar contractions of the nictitating membrane, and had comparable pressor effects, when injected intravenously into an anaesthetized cat. When measured as antagonists of acetylcholine, 10 μg. (−)-adrenaline equated with 0.1 ml. of the lactyladrenaline solution on the rat colon (middle record) and with 0.2 ml. on the rat uterus (bottom record). It was also noticed that rat tissues which were abnormally insensitive to adrenaline were also insensitive to lactyladrenaline (middle record).

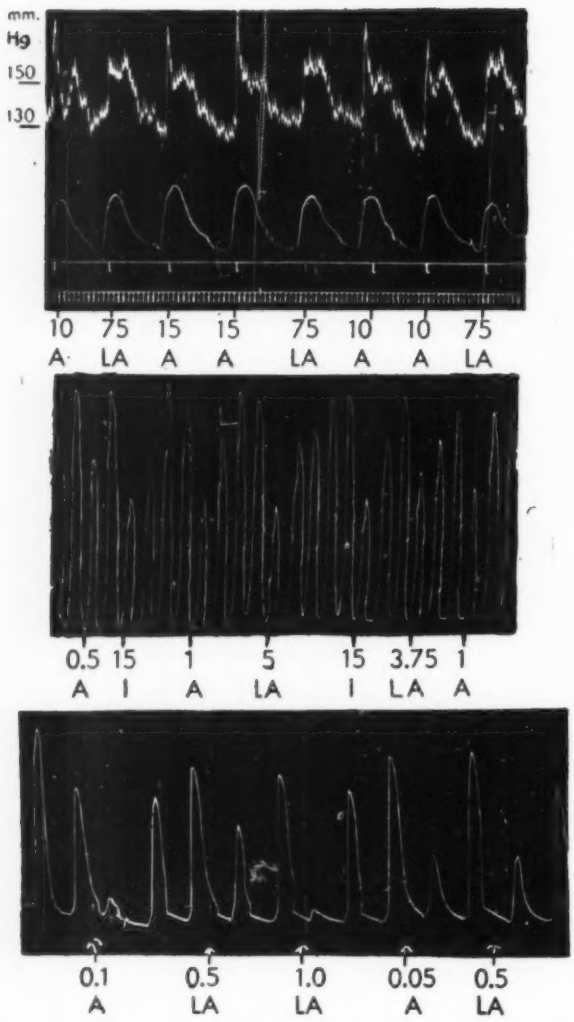

FIG. 5.—Comparison of adrenaline, lactyladrenaline, and isoprenaline. *Top record*, arterial blood pressure and contractions of nictitating membrane (cat under chloralose). *Middle record*, rat colon. *Bottom record*, rat uterus. Contractions of colon and uterus in response to a fixed dose of acetylcholine, and inhibited by the addition of other drugs. A, (−)-adrenaline in μg., LA, lactyladrenaline in μg., I, (±)-isoprenaline in ng.

DISCUSSION

The evidence presented has clearly differentiated the newly discovered third amine both from the medullary hormones (adrenaline and noradrenaline), and from those artifacts (lactyladrenaline and lactylnoradrenaline) which are known to arise in extracts of adrenal glands.

The evidence also indicates that the third amine is not an artifact. Firstly, it was found only in the glands of certain species, although identical mild extraction and separation processes were applied to those of all the species. Secondly, were third amine produced as an artifact, some correlation would be expected either between the total weight of amine extracted from the gland and submitted to chromatography, or between the adrenaline/noradrenaline ratios of the glands, and the incidence of third amine: no such correlation existed. Finally, the very great pharmacological potency of this third amine argues against the artifact hypothesis.

The colour reactions given by the third amine indicate that it is a catechol amine, containing either primary or secondary amino nitrogen and a free hydroxyl group in the side chain. The type of pharmacological action shown by this amine resembles, in general, that of noradrenaline with a single *N*-substituent of chain length equal to or exceeding two carbon atoms (Konzett, 1940b). In particular, the great potency of the third amine as an antagonist of pilocarpine-induced bronchospasm indicates a possible identification with isoprenaline (Konzett, 1940a). This last hypothesis receives great support from the results of a series of parallel quantitative assays which failed to differentiate the third amine from isoprenaline. The acceptance of this identification reveals a hitherto unknown biochemical synthesis, which remains to be investigated.

SUMMARY

1. A hitherto unreported sympathomimetic amine has been detected in saline extracts of adrenal glands from cats, monkeys, and man.

2. This amine was separated chromatographically, and was present only in trace quantity. It has been shown to differ in R_F value, and in pharmacological activity, from adrenaline, noradrenaline, and their lactyl derivatives.

3. The third amine resembles isoprenaline very closely in R_F value, colour reactions, and pharmacological activity. It could not be differentiated from isoprenaline in parallel assays.

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It is a pleasure to thank Miss Ann Hommersham for her skilled assistance.

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